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Control of Intestinal Spirochaete Infections in Chickens

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by
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Foreword

Infection of layer and broiler breeders with anaerobic intestinal spirochaetes (Avian Intestinal Spirochaetosis: AIS) has emerged as a widespread and potentially important constraint to optimal production in both the Egg and Chicken Meat Industries in Australia. The microrganisms involved are difficult to isolate and characterise, with the consequence that diagnosis of AIS is difficult. Moreover, there is a lack of information about how to control these infections.

The purpose of the present study was to help control AIS in Australia through a number of initiatives designed to improve capacity, and to acquire background information for use in more detailed future application of the techniques in the field. These procedures included provision of specialised diagnostic services, including development and evaluation of improved molecular means to identify the organisms, and to type individual strains that might be involved in particular flocks. An examination of *in vitro* drug sensitivities of Australian isolates of the organisms also was undertaken, to give a better understanding of what antimicrobial drugs might be effective for control. Finally, experiments were devised to examine the pathogenic potential of the organisms in adult birds, and to determine whether antimicrobials or enzyme addition to the diet might assist in control of AIS.

In this report the results of analysis of diagnostic submissions, evaluation of a new polymerase chain reaction technique for identifying *Brachyspira intermedia*, development of pulsed field gel electrophoresis for strain identification of *B. intermedia*, and multilocus enzyme electrophoresis analysis for examining overall relationships amongst Australian strains of avian intestinal spirochaetes are reported. Results of *in vitro* susceptibility of isolates to antimicrobial drugs are also recorded. Finally the results of experimental infection of layer and broiler breeder hens with *B. intermedia* and *B. pilosicoli* strains are documented, together with the outcomes of preventive treatments with antimicrobials or the addition of exogenous dietary enzyme in experimentally infected birds.

This project was funded from industry revenue which is matched by funds provided by the Federal Government.

This report, a new addition to RIRDC's diverse range of over 700 research publications, forms part of our Chicken Meat and Eggs R&D programs, which aim to support increased sustainability and profitability in the chicken meat industry by focusing research and development on those areas that will enable the industry to become more efficient and globally competitive and that will assist in the development of good industry and product images, and to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

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Abbreviations

AIS: avian intestinal spirochaetosis

ET: electrophoretic type

MIC: minimum inhibitory concentration

MLEE: multilocus enzyme electrophoresis

PCR: polymerase chain reaction

PFGE: pulsed field gel electrophoresis

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Executive Summary

Background

Over the past 15 years intestinal spirochaetal bacteria of the genus *Brachyspira* (formerly *Serpulina*) gradually have become recognised as common potential pathogens of commercial layer and broiler breeder chickens. The role of species of these bacteria as important pathogens of pigs has long been accepted. The organisms have fastidious growth requirements and need anaerobic conditions for their laboratory isolation: this complicates diagnosis and helps explain why it has taken so long for their significance to be appreciated in poultry. Several distinct species of these bacteria infect poultry, but not all of these are pathogens. Despite an increased awareness of intestinal spirochaete infections in chickens (Avian Intestinal Spirochaetosis: AIS), relatively few studies have been carried out on the condition.

Studies in Europe and the United States have found that infection of the caeca with intestinal spirochaetes is associated with the occurrence of wet droppings, delayed onset of egg laying, faecal staining of eggshells, reduced egg weights and reduced carotenoid content of eggs. Broiler chicks hatched from infected hens show reduced weight gain.

In Australia, studies of intestinal spirochaetal infection in commercial poultry flocks have shown that colonisation with these organisms is common. Moreover, spirochaetes are recovered significantly more frequently from layer and broiler breeder flocks with diarrhoea and reduced egg production than from clinically normal flocks. The results of pathogenicity trials in broilers and layers has shown that Australian strains of both *Brachyspira intermedia* and *B. pilosicoli* isolated from chickens have the capacity to cause disease and loss of production. Despite these findings, little is currently known about how to control the infections.

The current study was a collaborative effort involving Professor David Hampson and his colleagues at Murdoch University in Western Australia and Carol Stephens and her colleagues at the Toowoomba Veterinary Laboratory in Queensland. The study was undertaken with the aim of providing means to help control the infections. The objectives that were designed to achieve this involved improving the diagnostic infrastructure for AIS that is available in Australia, including evaluating new molecular diagnostic and strain typing techniques for the organisms, by assessing their *in vitro* antimicrobial sensitivity, and by testing antimicrobials and a commercial exogenous enzymes for their effectiveness in treating the infections in experimentally-infected layers and broiler breeders. The study also had the added benefits of improving collaboration between the laboratories, and providing a project through

which Carol Stephens will obtain a PhD degree in the area through the Division of Veterinary and Biomedical Sciences at Murdoch University.

Diagnostic submissions

Over the two years of the study, diagnostic submissions for suspected AIS were received from 36 layer or broiler breeder flocks, most of which were in Queensland. A positive diagnosis of AIS was made in 13 cases (28%), with both *B. intermedia* and *B. pilosicoli* identified as being the major agent involved. The isolates were examined further in other parts of the project.

Polymerase chain reaction (PCR) for identification of *Brachyspira intermedia*

A polymerase chain reaction assay amplifying a portion of the 23S rDNA gene was evaluated for identification of *B. intermedia.* A total of 34 strains of *B. intermedia* isolated from chickens and pigs and 195 strains of other related spirochaete species were tested. The optimised assay correctly identified all the *B. intermedia* strains, but generated 11 false positive reactions, giving a test sensitivity of 100% and a test specificity of 94.3%. The new PCR is a useful addition to the diagnostic armoury for identifying intestinal spirochaete isolates from chickens.

Pulsed field gel electrophoresis (PFGE) for strain typing of *Brachyspira intermedia*

Pulsed field gel electrophoresis was developed to investigate diversity amongst 34 *B. intermedia* strains from chickens and pigs. All strains had distinct DNA banding patterns, although three isolates from chickens on the same farm appeared closely related. The strains showed considerable genetic diversity. The most closely related chicken and pig strains shared only 62% similarity. These results suggest that transmission of strains between chickens and pigs is not very likely. The typing technique itself will prove useful for studying transmission of strains within and between flocks.

In vitro antimicrobial sensitivities of avian intestinal spirochaetes

The susceptibilities of 80 intestinal spirochaete isolates from chickens was tested *in vitro* against seven antimicrobial drugs that are known to inhibit growth of intestinal spirochaetes from pigs. There were some spirochaete species differences in their susceptibility to the drugs, but in general there was a high degree of susceptibility of the isolates to most of the drugs. This is fortunate since it means that there are antimicrobials available that potentially can be used to treat AIS in Australian flocks.

Multilocus enzyme electrophoresis for establishing genetic relationships between avian intestinal spirochaetes

Sixty two intestinal spirochaete isolates, including 56 from 15 Australian flocks, were analysed using multilocus enzyme electrophoresis (MLEE). The work was undertaken to help support the species identification of chicken isolates, and in particular to help assess the sensitivities and specificities of the PCR techniques being used and developed in the project. The study also provided additional information about the genetic relatedness and diversity of intestinal spirochaete isolates from Australian chickens, and compared these with non-Australian isolates.

For *B. pilosicoli* isolates there was a perfect correlation between MLEE grouping (m) and PCR results, with 16 isolates from five flocks divided into 6 electrophoretic types (ETs). The 13 *B. intermedia* isolates were defined by their location in MLEE group g. Of these 13, only four were positive in the *nox* PCR, but all were positive in the new 23S rRNA PCR. The isolates were from six Australian flocks, and belonged to 11 ETs. As with *B. pilosicoli*, strains from the same farm differed in some cases. Hence multiple strains of these species may be present in certain flocks, and this may make treatment more difficult if they have different drug sensitivities or vary in some other important properties. The *nox* PCR for the combined species *B. innocens*/*B. murdochii* was positive with nine isolates. Seven of these belonged to MLEE group d, identified as *B. murdochii*, one was in group b (a new unnamed group), and one in group a (also new and unnamed). Ten other isolates in group d (*B. murdochii*) were negative in the PCR, as were three in group e (*B. innocens)*. These results indicate that the *B. murdochii*/*B. innocens nox* PCR is specific, but has poor sensitivity for detection of these two species. This probably does not present a major diagnostic problem, since neither species is considered pathogenic in poultry. However, these isolates all came from flocks with wet litter or production problems, and it is still necessary to clarify their pathogenic potential by testing them under experimental conditions.

Overall, eight new genetic groups of intestinal spirochaetes were identified by MLEE, although each was only represented by one or two isolates. Further work is required to clarify whether these groups represent new species, and whether they have any significance in relation to disease production. No isolates of *B. alvinipulli* were identified amongst the Australian isolates. To date *B. alvinipulli* has only been isolated from a single diseased flock in the USA (Swayne *et al*., 1995).

Experimental infections of layer hens and response to antimicrobials and enzyme

Three experiments were conducted, with the aim of determining useful means of treating layer hens with AIS.

Experiment 1

Thirty individually caged layer hens were inoculated with *Brachyspira intermedia*, and 20 control birds remained unchallenged. Birds received a diet containing 100 ppm zinc bacitracin (ZnB), and were monitored for ten weeks. *B. intermedia* was recovered sporadically from five of the inoculated birds, and there were no significant effects on body weight, faecal water or egg production. ZnB was presumed to be inhibiting spirochaete growth, and when removed from the diet, 18 of the 30 inoculated birds rapidly became culture positive. After 4 weeks, 10 of the 30 infected birds were treated with tiamulin at 25mg/kg for 5 days, and 10 were returned to the diet containing ZnB. Birds receiving tiamulin became spirochaete negative, and maintained their egg production, but reinfection occurred. The other 20 infected birds had a significant drop in egg production, but those receiving ZnB showed a reduced colonisation by *B. intermedia* after three weeks. This experiment demonstrates that ZnB at 100ppm in the diet inhibits the growth of *B. intermedia.* Treatment of infected birds with tiamulin is effective, but birds can rapidly become reinfected. The study also confirms that *B. intermedia* infection in layer hens can reduce egg production.

Experiment 2

Brachyspira intermedia strain HB60 was used to experimentally infect 40 individually caged 22-week-old laying hens. Another 10 control birds were sham inoculated with sterile broth. All chickens received an experimental layer diet based on wheat. The infected birds were randomly divided into four groups of 10, with the diet for each group containing either 50 ppm zinc bacitracin (ZnB), 100 ppm ZnB, 256g/tonne of dietary enzyme (Avizyme®, 1302), or no additive. Birds were kept for six weeks after infection, and faecal excretion of *B. intermedia*, faecal water content, egg numbers, egg weights and body weights were recorded weekly. Control birds remained uninfected throughout the experiment. *B. intermedia* was isolated significantly less frequently from the groups of experimentally infected birds receiving ZnB at 50 ppm or Avizyme®, than those receiving 100 ppm ZnB, or no treatment. Infected birds had a transient increase in faecal water content in the week following challenge, but no other significant production differences were detected amongst the five groups of birds in subsequent weeks. It was not established how the ZnB at 50 ppm and the dietary enzyme reduced the ability of the spirochaete to colonise, but it may have been by bringing about changes in the intestinal microflora and/or the intestinal microenvironment.

Experiment 3

Brachyspira pilosicoli strain CPSp1 isolated from a chicken in a broiler breeder flock in Queensland was used to experimentally infect 40 individually caged 22-week-old laying hens. Another 10 birds were sham inoculated with sterile broth. All chickens received a commercial layer diet, but 10 infected birds had 50ppm ZnB incorporated in their food. Birds were kept for seven weeks, and faecal moisture, egg numbers, egg weights and body weights were recorded weekly. *B. pilosicoli* was isolated from the faeces of only three of the 30 inoculated birds receiving the diet without ZnB, whereas seven of the 10 inoculated birds receiving ZnB in their diet were colonised. This difference in colonisation rate was highly significant (P =<0.001). Dietary ZnB at 50 ppm therefore predisposed to colonisation by *B. pilosicoli.* Despite colonisation, no significant production differences were found between the birds in the three groups.

Experimental infection of broiler breeders and response to antimicrobials

Two experiments were conducted to investigate the pathogenic potential of *B. pilosicoli* in adult hens (broiler breeders), and to determine whether the infection could be treated with two antimicrobials commonly used to treat intestinal spirochaete infections in pigs (tiamulin and lincomycin).

Experiment 1

The pathogenic potential of *Brachyspira pilosicoli* and *Brachyspira innocens* was evaluated in thirty 17-week-old Cobb broiler breeder hens individually caged in three groups of 10 birds. Control birds were sham inoculated with sterile broth medium. Birds in the other two groups were inoculated respectively with an isolate of *B. innocens* or of *B. pilosicoli*. Birds were monitored daily, and killed at 41 weeks of age. Infection had no consistent effect on body weight gain, but inoculation with *B. pilosicoli* resulted in a brief increase in faecal water content. *B. innocens* had no effect on egg production, but *B. pilosicoli* infection caused a delayed onset of laying, and a highly significant reduction in egg production over the first 11 weeks of lay. This study confirms that *B. pilosicoli* can cause serious egg production losses in adult chickens, whilst *B. innocens* is non-pathogenic.

Experiment 2

Brachyspira pilosicoli strain CPSp1 was used to experimentally infect 30 individually caged 22-week-old Cobb 500 broiler breeder hens. Another 10 birds were sham inoculated with sterile broth. The birds failed to become colonised. At 29 weeks of age they were transferred to a diet containing 50 ppm ZnB and were rechallenged with the *B. pilosicoli* strain at 32 weeks of age, weekly for five weeks. The majority of the birds then became colonised. Ten birds were then treated by crop tube with 25 mg/kg body weight tiamulin for five days, and 10 with 20mg/kg body weight lincomycin for five days. Both

these treatments removed the infection, whilst untreated birds remained infected. The results of this experiment support previous observations that ZnB at 50 ppm in the diet increases the susceptibility of birds to *B. pilosicoli* infection. The study also demonstratess the usefulness of both tiamulin and lincomycin for treatment of infection with *B. pilosicoli* in adult birds.

Implications

The fact that regular diagnoses of AIS were made in layer and breeder flocks with production problems throughout this two year project emphasises that infection with intestinal spirochaetes is a real problem to the Industries in Australia. The condition is undoubtedly going undiagnosed in many cases due to insufficient awareness on the part of veterinarians and pathologists and a lack of expertise and facilities in diagnostic laboratories around Australia. Clearly both the Industries and the laboratories that service them need to be more aware of AIS as a possible differential diagnosis where wet litter and production drops are involved, and of what facilities are available for diagnosis. The molecular identification and strain typing techniques developed during this project will improve diagnostic capacity in Australia.

In general the spirochaete species remain susceptible to a range of antimicrobial agents, although resistance against tylosin was evident for a number of strains. The different spirochaete species tended to have a different range of sensitivities to some of the drugs tested. Clearly antimicrobial sensitivities need to be monitored on an ongoing basis.

The pathogenicity studies in adult birds confirmed that both *B. intermedia* and *B. pilosicoli* are capable of causing loss of production, although complex interactions which may modulate disease expression appear to occur between the spirochaetes and the intestinal microflora. In particular, in-feed zinc bacitracin increased the susceptibility of birds to *B. pilosicoli* infection, although it offered protection against *B. intermedia*. These differences emphasise the need for good diagnostic capacity to identify the spirochaete species involved in individual cases of AIS. Furthermore, research is required to understand the basis of these effects, so that improved control measures can be developed.

The study demonstrated that both tiamulin and lincomycin can be used to treat AIS. Both drugs are effective, but a problem arises from the potential for birds to become reinfected after treatment has ceased. Tiamulin has recognised incompatibilities with the use of ionophores (for control of cocidiosis), and this incompatibility would need to be considered before any treatment was undertaken. As most of the antibiotics used in this work require veterinary prescription and many are not registered for use in egg laying poultry, producers should seek veterinary advice before using any antibiotics to control intestinal spirochaetes.

Finally, the observation that addition of xylanase to the diet can reduce colonisation by *B. intermedia* has important ramifications. The mechanisms behind this require further study, but presumably may be linked to changes in viscosity of the intestinal digesta. If this is the case, there are also likely to be dietary influences on AIS – although the possibility of different responses for *B. intermedia* and *B. pilosicoli* does need to be investigated.

Recommendations

The current findings should be disseminated to chicken producers and their veterinarians in Australia. In particular the potential for zinc bacitracin to increase susceptibility to *B. pilosicoli* should be made known. Similarly the potential beneficial effects of dietary xylanase preparations in reducing colonisation by *B. intermedia* in adult birds should be made known to the Industries.

Research funds should be made available for future research:

- to determine the mode of action of the xylanase in reducing colonisation by *B. intermedia*, and determining whether the same effect occurs with *B. pilosicoli*
- to investigate the mechanism whereby zinc bacitracin increases susceptibility to colonisation by *B. pilosicoli*
- to investigate the potential influence of diet on infection with intestinal spirochaetes in chickens.
- to study the epidemiology of the infections in depth: specifically it is necessary to find out how infection arises in a shed, how transmission occurs between birds and sheds, and what reservoirs of infection may exist. This can be supported by the methodologies for strain typing that have now been developed
- to investigate the potential for chicken isolates of *B. pilosicoli* to cause disease in humans

1. Introduction

1.1. Background

This study aimed to apply knowledge gained from working with related pathogens of pigs to develop approaches to control infections with the intestinal spirochaetes *Brachyspira (Serpulina) intermedia* and *Brachyspira (Serpulina) pilosicoli* in Australian poultry. Both are newly described pathogenic species of intestinal spirochaete, members of which also infect pigs (Hampson and Trott, 1995; Trott *et al*, 1996c; Stanton *et al*, 1997). Infection with pathogenic intestinal spirochaetes in chickens has been termed Avian Intestinal Spirochaetosis (AIS) (Swayne, 1997).

The possible importance of intestinal spirochaetes of the genus *Brachyspira* (formerly *Serpulina*) as pathogens in poultry was first shown in the Netherlands. Davelaar and colleagues (1986) demonstrated spirochaetes in the caecal mucosa of laying hens with diarrhoea, and used these to induce typhlitis in ten-week-old hens, with an associated increase in faecal water content and a retardation of growth. Subsequently, Griffiths and colleagues (1987) in the UK described a similar condition in pullets, with reduced growth rate, delayed onset of egg production, and poor shell quality. Dwars and colleagues (1987) investigated prevalence of infection in Dutch poultry flocks and demonstrated spirochaetes in the caecae of birds from 37 of 134 (27.6%) flocks with intestinal disorder, but in only two of 45 (4.4%) flocks with no signs of enteritis.

Dwars and colleagues (1992b) used their unclassified Dutch spirochaete isolate 1380 in a series of experimental infections of day-old-chicks. Infection resulted in depression of growth, decreased serum concentrations of protein, lipid carotenoids and bilirubin, and an increased faecal fat content. Infection of layer hens increased their faecal lipids (Dwars *et al*. 1992a), whilst infection of broiler breeders resulted in wet faeces, reduced egg production and weight, and reduced growth of broilers hatched from their eggs (Dwars *et al*., 1993). We subsequently showed that this organism is *B. intermedia* (McLaren *et al*., 1997). Natural infection of pigs with this organism occurs infrequently, causing a grey-green diarrhoea (Hampson and Trott, 1995).

In the USA, Swayne and colleagues (1992) isolated spirochaetes from cases of typhlitis in commercial layer flocks. Affected birds had pasty vents, and associated staining of egg shells with faeces made the eggs unsuitable for table consumption. Working with Dr Swayne, we were able to show that these spirochaetes belonged to a new group which had not previously been described (Swayne *et al*, 1995; McLaren *et al*., 1997). Subsequently these organisms were assigned to the new species *Brachyspira alvinipulli* (Stanton *et al.,* 1998). In Iowa, Trampel and colleagues (1994) described intestinal spirochaetes colonising the caecae of laying hens with a 5% reduction in egg

production. These spirochaetes characteristically attached by one cell end to the caecal mucosa. In subsequent work we showed that their isolate 308.93 was *B. pilosicoli* (McLaren *et al*., 1997). This organism is a common and important pathogen of pigs, causing a condition called "porcine intestinal spirochaetosis" (Trott *et al*., 1996b,c). This species also infects other species, including dogs, wild birds and human beings (Trott *et al*., 1996a; Hampson and Stanton, 1997; Oxberry *et al.,* 1998).

Supported by funds from the RIRDC Chicken Meat and Egg Programs, we undertook a study to determine the prevalence of intestinal spirochaetes in flocks in Australia. Using selective media in anaerobic conditions we cultured 410 faecal samples from 37 layer flocks and 257 faecal samples from 30 broiler breeder flocks (overall 10-40 samples per flock) in Western Australia (WA) (McLaren *et al*., 1996). We isolated spirochaetes from 13 of the layer flocks (35.1%) and 16 (53.3%) of the broiler breeder flocks. Spirochaetes were isolated from 64% of flocks recording production problems, including wet litter, and from 28% of apparently healthy flocks. This difference was statistically significant ($P<0.02$).

Analysis of these spirochaetes using multilocus enzyme electrophoresis (MLEE), identified 38.5% as being *B. intermedia*, 56.4% as belonging to a previously undescribed and unnamed group ("*Brachyspira pulli*") and the rest being *Brachyspira innocens* (a non-pathogenic species in pigs) (McLaren *et al*., 1997). No isolates of *B. alvinipulli* or *B. pilosicoli* were made in the WA survey. To follow up these findings we then conducted a prevalence survey of intestinal spirochaetes in poultry flocks in the eastern states of Australia. Faecal samples (n=1786) from chickens in broiler (n=19), broiler breeder (n=28) or layer (n=22) flocks were cultured for intestinal spirochaetes. Overall, 42.9% of broiler breeder and 68.2% of layer flocks were colonised with spirochaetes, but no broiler flocks were infected. Colonisation rates in infected flocks ranged from 10 to 100% of birds sampled. Faeces from colonised flocks were on average 14% wetter than those from non-colonised flocks. There was a highly significant association between colonisation with spirochaetes and the occurrence of wet litter and/or reduced production. A subset of 40 spirochaete isolates were identified to the species level using a panel of polymerase chain reaction tests. Seven (17.5%) of these isolates were identified as *B. intermedia* whilst 13 (32.5%) were *B. pilosicoli*. The common isolation of *B. pilosicoli* was in contrast to the WA findings, and demonstrates that the overall situation in Australia is even more complex and problematic than had been anticipated. Furthermore half of all the isolates belonged to two species that are known pathogens of chickens.

Three of the WA isolates of *B. intermedia*, three from the unnamed group and a *B. pilosicoli* isolate from Queensland were used to infect day-old broiler chicks, which were kept for three weeks. Chicks infected with the *B. intermedia* strains developed diarrhoea 7-9 days post-inoculation, and were significantly lighter than the control birds on weeks 1 and 3 post-inoculation. Birds infected with *B. pilosicoli* developed diarrhoea 7-9 days post-inoculation, and were significantly lighter than the

controls on weeks 1 and 2 post-inoculation. Chicks infected with strains of the unnamed Western Australian group developed diarrhoea 12-13 days after inoculation, but their weights were not significantly depressed. No consistent pathological changes were found in the caecae of any of the birds, apart from end-on attachment with *B. pilosicoli*.

WA *B. intermedia* strain HB60 was also used to infect a group of ten 14-week-old hens. Nine birds became colonised and remained so for the 16 week duration of the experiment. Infected birds tended to be lighter than the controls throughout, and had less body fat at slaughter. Infected birds had wetter and less well-formed faeces, with a significant increase in faecal moisture content overall (from 77.13% to 80.14%: $P<0.0001$). Over the period 7-15 weeks post-infection, infected birds produced an average of 0.503 eggs per day compared to 0.670 for the uninfected controls. This difference was statistically significant ($P<0.05$). The average weight of the eggs produced by infected birds was also significantly less ($P<0.001$) than the control birds (44.5g compared to 45.36g) (Hampson, 1997; Hampson and McLaren, 1997, 1999).

Overall, these studies helped confirm that infections with intestinal spirochaetes are a common but currently under-diagnosed cause of wet litter and/or reduced egg production in broiler breeder and layer flocks in Australia.

Remarkably, no specific recommendations concerning control of intestinal spirochaete infections have been made, and there have been very few reports on treatment of intestinal spirochaete infections in chickens. Swayne (1997) suggested that antimicrobials used for the treatment of *B. hyodysenteriae* (the agent of swine dysentery) would be useful in chickens, and also reported the successful use of neomycin to prevent clinical signs of AIS. Antimicrobial sensitivity testing of two US chicken isolates of *B. pilosicoli* and two of *B. alvinipulli* in agar dilution showed that they had high susceptibility to tiamulin, lincomycin and carbadox (drugs used for swine dysentery), but gave highly variable results for a number of other antimicrobials (Trampel *et al.*, 1999). In the Netherlands, Smit and colleagues (1998) described the treatment of naturally infected hens with a six day course of 120 ppm of a 5-nitroimidazole compound in the drinking water. Treatment before the onset of lay prevented the negative effects of spirochaetes on egg production, but later treatment was less effective, and had only a temporary effect. This may have been due either to reinfection of the birds from contaminated litter, or to incomplete treatment of all the birds in the flock.

Stephens and Hampson (1999) reported treating two meat breeder flocks that were infected with a mixed population of intestinal spirochaetes and were exhibiting reduced production. Colonisation rates in these flocks were 100% and 73% of sampled birds respectively. The first flock was treated with lincospectin in the water for seven days at a rate of 50 mg per bird per day. The second flock was treated with tiamulin at 25 mg per kg bodyweight in the water for five days. Following

antimicrobial treatment, the condition of the birds in both flocks improved slowly. Treatment with lincospectin resulted in slimy faeces, which lasted for two to three weeks. Birds in this shed remained spirochaete negative for almost three months, at which time 30% of samples were culture positive. This level of colonisation continued for four months. Following treatment with tiamulin, spirochaetes were no longer detectable for three weeks, after which they started to be detected at a low prevalence. This prevalence continued for almost three months, then increased to 80%. These observations again indicated that re-infection with spirochaetes may be a problem, particularly in birds heavily infected at the time of treatment. The possible occurrence of mixed infections with different spirochaete species or different strains within the flocks also may have made antimicrobial treatment problematic.

1.2. Resources and needs

As a cornerstone for control of AIS in Australia, we believe it is important to maintain expertise in specific laboratories where these fastidious organisms can be isolated and identified on behalf of producers and the diagnostic laboratories. The laboratories at Murdoch University and at Toowoomba both have this capacity. We have already developed rapid polymerase chain reaction (PCR) technology for identifying *B. pilosicoli* based on the 16S rRNA gene (Park *et al*., 1995), and a similar test for *B. intermedia* strains based on the NADH Oxidase (*nox*) gene (Atyeo *et al.,*1999). Unfortunately the latter test is not particularly sensitive for avian strains of *B. intermedia*, so as part of the current project it was proposed to develop and test an alternative PCR for *B. intermedia* based on the 23S rRNA gene for its usefulness on a large number of *B. intermedia* strains from poultry. The availability of this capacity would be an important adjunct to rapid detection and identification of this species. In addition, we wished to apply multilocus enzyme electrophoresis (MLEE) and pulsed field gel electrophoresis (PFGE) as complimentary means of strain typing and assessing genetic diversity in isolates from poultry. We have already developed and used the techniques with considerable success for *B. pilosicoli* (Atyeo *et al*., 1996; Trott *et al*., 1998).

There is no published data on the antimicrobial drug sensitivity of *B. intermedia* and *B. pilosicoli* strains from chickens. Therefore we proposed testing a collection of Australian poultry strains of *B. intermedia* and *B. pilosicoli* against a panel of antimicrobial agents, in agar dilution, as we have previously done with Australian *S. hyodysenteriae* isolates. We would then test apparently effective and appropriate drugs in groups of adult birds experimentally infected with *B. intermedia* or *B. pilosicoli*. This was to develop a rapid first-line approach to the control of the infections.

As a component of the project we also wanted to investigate a means of control which did not rely on antimicrobials. Such an approach would avoid problems associated with consumer concerns over drug residues, and issues of withholding periods. Recently we have shown that swine dysentery can be completely prevented by dietary means (Siba *et al*., 1996; Pluske *et al*., 1996). Pigs fed diets low

in substrate which is rapidly fermented in the large intestine ("fibre": soluble non-starch polysaccharides [sNSPs] and resistant starch [RS]), completely resist experimental colonisation by *B. hyodysenteriae*. We believe this is mediated either via changes in the resistant microflora (whose activity is required to facilitate *B. hyodysenteriae* colonisation), or possibly by changes in the viscosity and/or hydration of the large intestinal contents. Levels of sNSP and RS can be reduced by appropriate choice of dietary ingredients (eg. sorghum contains less NSP than wheat), by physical processing (eg. extrusion to reduce RS), or by enzymatic treatment. Our original protective diet was based on cooked white rice and animal protein, with lesser levels of protection obtained with steam-flaked maize and sorghum. We are currently investigating extrusion and/or enzymatic treatment of wheat to develop protective pig diets.

Since *B. intermedia* and *B. pilosicoli* strains are genetically closely related to *B. hyodysenteriae*, and share the same ecological niche in the large intestine, we proposed investigating whether it is possible to prevent colonisation of poultry by these species by reducing the levels of sNSP and RS content by the addition of exogenous enzymes. Addition of enzymes has been shown to reduce problems of wet litter on certain farms, and it may be that this is mediated through effects on growth of pathogenic intestinal spirochaetes in the caeca.

2. Objectives and tasks

The overall aim of the project was to identify means to control infections by the intestinal spirochaetes *Brachyspira intermedia* and *B. pilosicoli* in Australian layer and broiler breeder flocks. To do this, three main objectives were devised:

1. To operate a diagnostic service to help improve diagnosis of intestinal spirochaete infections in chickens. As part of this service, to improve the infrastructure for controlling AIS, a new PCR technique for identifying *B. intermedia* would be evaluated, and pulsed field gel electrophoresis (PFGE) also evaluated for strain typing of *B. intermedia*. PCR and PFGE techniques had already been developed and evaluated for *B. pilosicoli*. Antibiotic sensitivities of Australian intestinal spirochaete isolates from poultry also would be tested.
2. To develop and use multilocus enzyme electrophoresis (MLEE) in conjunction with the tests described above to investigate the identity and relationships of intestinal spirochaetes from Australian chickens.
3. To conduct infection trials in layers and broiler breeders with the object of testing the efficacy of potential antimicrobial therapy and a dietary enzyme treatment for controlling intestinal spirochaetes.

3. Laboratory studies

3.1. Diagnostic services

Throughout the period of the project, diagnostic services specifically for detection and identification of avian intestinal spirochaetes were operated from the Toowoomba Veterinary Laboratory, and from the Division of Veterinary and Biomedical Sciences at Murdoch University. The Toowoomba laboratory is a Queensland state government veterinary diagnostic laboratory, and hence in the course of its normal operations regularly receives diagnostic submissions from poultry producers.

3.1.1. Diagnostic submissions to the Toowoomba Veterinary Laboratory

During the course of the project 30 accessions from commercial poultry flocks reportedly experiencing production problems consistent with AIS were examined. Clinical signs reported included depression, wet droppings and reduced egg production.

Necropsy was performed on any whole birds that were submitted, as part of a routine diagnostic investigation. Samples for histology were fixed in 10% buffered neutral formalin, paraffin-embedded and sectioned at 5 μm. All sections were stained with haematoxylin-eosin, while sections of caeca, if available, were stained with Steiner silver stain. When available, caecal contents or faeces were cultured for spirochaetes.

Of the 30 accessions examined, diagnoses other than spirochaetosis were made in 16 instances. These included Mareks, coccidiosis, fowl pox, egg peritonitis and other bacterial infections. No diagnosis was made in two accessions. A further accession, consisting of faecal samples only, was specifically submitted for spirochaetal culture from a commercial flock reporting wet droppings. The faecal samples were two weeks old and very dry when received and were negative on culture.

Spirochaetes were observed in histological sections and or were cultured from caecal contents or faeces from the remaining 11 accessions. Diagnoses of AIS were made where this was indicated by both histological and cultural results. Where only histological evidence of spirochaetes was available and the species could not be determined, a diagnosis of suspected spirochaetosis was made. A number of conditions, in addition to the presence of spirochaetes, were found in birds from six of the accessions. These included egg peritonitis, vent pecking, cannibalism, Mareks disease and mycoplasmosis.

Notable accessions

A submission of formalised caeci only, from a commercial flock of meat breeders experiencing diarrhoea of insidious onset. Histological examination of ten caeca from birds of 39 weeks of age from four high bio-security sheds each housing 2500 birds, revealed six caeca with moderate to high numbers of spirochaetes. Eight of ten caeca from a group of 34 week old birds had low numbers of spirochaetes, while a further two of ten caeca from another group of 31 week old birds from this flock were also positive for spirochaetes.

An accession was received from a commercial layer operation consisting of four, 34 week old birds from a group of 20,000 that was experiencing wet droppings and vent soiling associated with egg drop. Histological examination of the caeca from these four birds revealed that all contained moderate to large numbers of spirochaetes invading the crypts. *Brachyspira intermedia* was isolated from all four caeca. A diagnosis of AIS was made.

A commercial layer farm experiencing up to 25% drop in egg production submitted six birds for necropsy. Four of six caeca were culture positive for spirochaetes of unknown species.

Spirochaetes were also isolated from commercial free-range layers, of mixed age, ranging from 34 to 69 weeks. This farm had been experiencing a significant drop in egg production, associated with diarrhoea, for some months. Of ten faeces submitted for culture, two were positive for *B. intermedia*. A diagnosis of spirochaetosis, in association with tracheitis and helminth infestation, was made in this case.

A major commercial meat breeder operation experiencing a severe wet dropping problem as well as production drop submitted ten faecal samples from 41 week old birds. Two of the ten samples were positive for *B. pilosicoli*.

A series of submissions from a commercial layer farm had sections of caeca that were positive for spirochaetes. Of 12 accessions, 11 were culture positive for spirochaetes. Spirochaetes from seven of these accessions were identified by polymerase chain reaction (PCR) tests. Three were *Brachyspira pilosicoli* and two *B. intermedia*, while one accession was a mixed infection of the above two species, plus an unidentified strain. Spirochaetes from the remaining accession were identified as being either *B. innocens* or *B. murdochii*.

Histological examination of the caeca infected with *B. pilosicoli* revealed large numbers of spirochaetes, some forming a pseudo-brush border on the surface of the caecal epithelium. Sections of caeca infected with *B. intermedia* revealed moderate to large numbers of spirochaetes, some of which appeared to have invaded the caecal epithelium. No other enteric pathogens were obtained from these accessions.

3.1.2. Diagnostic submissions to Murdoch University

Faecal samples were submitted from five layer flocks with problems ranging from wet litter to reduced egg production. Two flocks were found to be heavily colonised with spirochaetes identified as *Brachyspira intermedia.* Medication of one of these flocks with 2kg/tonne chlortetracycline for one week resulted in a 5% increase in egg production, and a marked reduction in number of birds colonised with spirochaetes.

3.2 Evaluation of a polymerase chain reaction assay amplifying part of the 23S rRNA gene for Identification of *Brachyspira intermedia* isolates

3.2.1. Introduction

Brachyspira intermedia is a species of weakly haemolytic anaerobic intestinal spirochaete which colonises the large intestines of both chickens and pigs (Stanton *et al.,* 1997). These spirochetaes are closely related to *Brachyspira hyodysenteriae,* a strongly beta-haemolytic species which causes a severe mucohaemorrhagic diarrhoea of pigs called swine dysentery (Harris *et al.,* 1999). The type strain of *B. intermedia,* PWS/A^T, closely resembles *B. hyodysenteriae* in that it produces indole, and it shows similar or intermediate metabolic patterns of substrate utilisation between *B. hyodysenteriae* and the non-pathogenic weakly beta-haemolytic species *Brachyspira innocens* (Lemcke and Burrows, 1981). Other strains with the phenotypic properties of PWS/A^T have been described (Binek and Szynkiewicz, 1984; Lee *et al*., 1993; Fellstrom and Gunnarsson, 1995), and when analysed using multilocus enzyme electrophoresis (MLEE) and 16S rDNA sequence comparisons they were shown to constitute a distinct genetic group, which was provisionally designated "*Brachyspira intermedius"* (Lee *et al*., 1993; Stanton *et al*., 1996). Subsequently DNA-DNA reassociation assays confirmed that these organisms were distinct from other named species of intestinal spirochaetes, and this led to their official naming as *B. intermedia* (Stanton *et al*., 1997).

The type strain PWS/A^T was originally isolated from a pig with post-weaning diarrhoea (Hudson *et al*., 1976). There is some evidence from the field to suggest that *B. intermedia* can cause diarrhea in swine (Binek and Szynkiewicz, 1984; Fellstrom and Gunnarsson, 1995), and this condition has been called porcine spirochaetal colitis (Hampson and Trott, 1995; Taylor and Trott, 1997). Nevertheless a number of workers have failed to produce diarrhoea in pigs experimentally infected with porcine strains of *B. intermedia* (Hudson *et al*., 1975; Neef *et al*., 1994), and this has led to the general belief that *B. intermedia* is a non-pathogenic commensal of pigs (Taylor and Trott, 1997).

Whether or not these organisms are pathogens, it is still important to be able to rapidly identify and differentiate them from other known pathogenic intestinal spirochaetes that infect pigs.

In contrast to the situation in pigs, there is much stronger evidence to suggest that *B. intermedia* is a pathogen of chickens. Dwars *et al*. (1992a; 1992b; 1993) demonstrated that intestinal spirochete strain 1380, isolated from a Dutch chicken with diarrhoea, was capable of inducing diarrhoea and reduced growth rates in experimentally infected chickens. Subsequently McLaren *et al*. (1997) used MLEE to show that this organism was a strain of *B. intermedia*. Likewise, other strains of *B. intermedia* were found to infect around 10% of broiler breeder and layer flocks in Western Australia (WA), and to be associated with reduced egg production and diarrhoea (McLaren *et al*., 1996; 1997). One of these strains, HB60, has been used to experimentally infect layer hens in which it caused increased faecal moisture and reduced egg production (Hampson and McLaren, 1999). In summary, infection of adult chickens with *B. intermedia* strains appears to be a common but infrequently recognised cause of production problems.

Until recently the only method available to detect *B. intermedia* was anaerobic culture on selective agar followed by identification of weakly beta-haemolytic, indole positive spirochaetes (Lee *et al.,* 1993; Fellstrom and Gunnarsson, 1995). The only definitive method of identification was the use of MLEE (Lee e*t al*., 1993; McLaren *et al*., 1997). As a starting point for any study of the role of these organisms in disease of pigs and poultry, more rapid and reliable identification and diagnostic techniques are required. Recently a PCR test amplifying a 1027 base pair portion of the 23S rRNA gene of *B. intermedia* was developed (Leser *et al*., 1997). This was tested on three *B. intermedia* strains from pigs, and applied in the identification of five further porcine field isolates corresponding to *B. intermedia,* as well as 56 other isolates of *Brachyspira* spp. The PCR correctly identified all strains of *B. intermedia* and did not give any false positive reactions. A PCR based on the NADH oxidase gene (*nox*) of *B. intermedia* has also been developed in our laboratory, but unfortunately although it correctly identify 10 porcine strains of *B. intermedia* it only amplified DNA from four of 10 strains from chickens (Atyeo *et al*., 1999).

The purpose of the current study was to determine the specificity and sensitivity of the 23S rRNA gene PCR assay for *B. intermedia* using a large collection of spirochaetes, and including *B. intermedia* strains from both chickens and pigs. The assay was subsequently used in our laboratory to assist in identifying field strains of intestinal spirochaetes.

3.2.2. Materials and methods

Spirochaete strains and culture conditions

All strains used in the study were obtained as frozen stocks from the collection held at the Australian Reference Laboratory for Intestinal Spirochaetes, Murdoch University. The 34 *B. intermedia* strains tested with the PCR are listed in Table 1. They had previously been identified as *B. intermedia* on the basis of their weak beta-haemolysis, their indole production, their API-ZYM profile and their grouping in MLEE (Lee *et al*., 1993; McLaren *et al*., 1997). Eighteen strains were from chickens and 16 from pigs. Another 195 well-characterised strains of other *Brachyspira* species were tested by PCR. These included 77 strains of *B. hyodysenteriae*, 62 strains of *Brachyspira pilosicoli*, 17 strains of *B. innocens*, 12 strains of *Brachyspira murdochii*, 14 strains of avian spirochaetes in the MLEE group d of McLaren *et al*. (1997), which have been provisionally designated "*Brachyspira pulli*" (Stephens and Hampson, 1999), 10 strains of canine spirochaetes provisionally designated as "*Brachyspira canis*" (Duhamel *et al*., 1998), two strains of *Brachyspira alvinipulli*, and one strain of the human intestinal spirochaete *Brachyspira aalborgi*.

Frozen stocks of the spirochaetes were transferred to Kunkle's prereduced anaerobic broth (Kunkle *et al*., 1986), and grown on a rocking platform at 37°C for 3-5 d before further subculture. Growth and purity were monitored under a phase contrast microscope, and cells were harvested in mid-log phase. Cells were washed and resuspended in phosphate buffered saline (pH 7.2) prior to use.

Polymerase chain reaction assay

Initially whole cells of the spirochaetes were used in the PCR, as previously described (Atyeo *et al.*, 1998; 1999). A sterile toothpick was lightly touched to the surface of a packed cell pellet of the organisms held at -20°C in the bottom of a microfuge tube. These were then applied directly to the PCR reaction. Subsequently, for strains where false positive amplification had occurred (presumably as a result of overloading with bacterial cells), genomic DNA was extracted using the phenol-chloroform method as previously described (Turner *et al*., 1995), and approximately 100 ng from each strain was tested. The PCR incorporated a wax barrier division in a hot start protocol (chill out wax phase separator; MJ Research), and used the reaction conditions described previously (Leser *et al*., 1997), including an optimised primer annealing temperature of 60°C. Samples giving false positive reactions were retested at a primer annealing temperature of 63°C. All strains were tested at least twice. Amplified product was subjected to agarose gel electrophoresis, stained by soaking the gel in ethidium bromide (0.5μg/ml) in distilled water, and viewed over a UV transilluminator.

Table 1: *Brachyspira intermedia* strains tested by PCR, their geographical origin according to Australian state or country, species of origin and health status of the animal from which they were isolated.

Strain	Geographical origin	Species of origin	Health status[a]
2818.5	Tasmania	Pig	Diarrhoea
Q90-21643	Queensland	Pig	NA
N96.2968.3	New South Wales	Pig	NA
Wesp 1584	Western Australia	Pig	NA
Of5	Western Australia	Pig	NA
An520.93	Sweden	Pig	Diarrhoea
Uni-4	USA	Pig	NA
2818.1	Tasmania	Pig	Diarrhoea
Q90.1673	Queensland	Pig	NA
V992.2f	Victoria	Pig	NA
W16	Victoria	Pig	NA
An983.90	Sweden	Pig	Diarrhoea
PWS/A^T	England	Pig	Diarrhoea
889	New South Wales	Pig	Diarrhoea
T94.6363	Tasmania	Pig	NA
V2173.19	Victoria	Pig	NA
B37iii	Western Australia	Chicken	NA
22/5	Western Australia	Chicken	Wet litter
22/6	Western Australia	Chicken	Wet litter
22/8	Western Australia	Chicken	Wet litter
3B-1	Western Australia	Chicken	Low production
P3	Western Australia	Chicken	NA
1380	The Netherlands	Chicken	Wet litter
E2	Western Australia	Chicken	Low production
A7	Western Australia	Chicken	Low production
Abb60-9	Western Australia	Chicken	NA
B52iii	Western Australia	Chicken	NA
Histo6	Western Australia	Chicken	Low production
APWG33	Western Australia	Chicken	Low production
B230	England	Chicken	Low production
2A-10	Western Australia	Chicken	Low production
HB60	Western Australia	Chicken	Wet litter
Histo5	Western Australia	Chicken	Low production
APWG34	Western Australia	Chicken	Low production

[a]NA, information not available.

3.2.3. Results

The PCR assay optimised at 60°C correctly identified all 34 of the *B. intermedia* strains, producing a product of the predicted 1027 base pair (bp) size. Of the 195 other spirochaete strains tested, 11 reacted in the PCR at an annealing temperature of 60°C, and all these generated product of 1027 bp size. The same results were obtained when the PCR was repeated with purified DNA from the same strains. This result gave the test an overall test specificity of 94.37%. Raising the annealing temperature to 63°C resulted in generation of false negative reactions with some *B. intermedia* strains, and resulted in only a marginal improvement in specificity. The false positive reactions were generated from five of the 77 (6.5%) *B. hyodysenteriae* strains, two of the 62 (3.2%) *B.*

pilosicoli strains, one of the 17 (5.9%) *B. innocens* strains, and three of the 14 (21.4%) *"B. pulli"* strains. The designations and origins of these strains are presented in Table 2.

Table 2: Strains of *Brachyspira* species other than *B. intermedia* that amplified in the 23S rDNA PCR

Species	Strain name	Species of origin	Place of origin
B. hyodysenteriae	B169	Pig	Canada
	B69933	Pig	Canada
	JWPM 300/8	Pig	The Netherlands
	3391-90b	Pig	Victoria, Australia
	3821	Pig	Victoria, Australia
"B. pulli"	Rag Heal	Chicken	The Netherlands
	PHB-9	Chicken	Western Australia
	APWG-35	Chicken	Western Australia
B. pilosicoli	Unl-2	Pig	USA
	Of2	Pig	Western Australia
B. innocens	An916.9	Pig	Sweden

3.2.4. Discussion

The PCR assay was 100% sensitive in terms of its identification of *B. intermedia* strains from both pigs and chickens, and this was in contrast to a previously described *nox*-based PCR which had a sensitivity of 70%, detecting all 10 porcine strains but only four of 10 chicken strains tested (Atyeo *et al*., 1999). The current assay had a test specificity of 94.3% when used with a large number of strains of related species, and although this was reasonably good it still could create problems for Veterinary Clinical Microbiology Laboratories. Eight of the false positive reactions were generated from porcine strains. Fortunately in the case of analysis of porcine spirochaetes the problem of lack of specificity would not be too important as the PCR for *B. intermedia* is only likely to be applied to confirm results in situations where PCRs for pathogenic *B. hyodysenteriae* and *B. pilosicoli* are also being used, and have proved negative (Atyeo *et al*., 1998; 1999). For chicken isolates the frequent amplification of the common presumed non-pathogenic species *"B. pulli"* may create a greater problem, giving the impression that chickens were infected with pathogenic *B. intermedia.* In this case it would be advisable to undertake an indole test on any positive isolate. Indole production is a constant feature of all *B. intermedia* strains, whilst it was only found in one of 25 *"B. pulli"* strains tested (McLaren *et al.,* 1997).

It was surprising that strains of four different spirochaete species other than *B. intermedia* all generated PCR product of the correct predicted size. Increasing the primer annealing temperature of the reaction did improve specificity, but only at the expense of sensitivity of detection of *B. intermedia* strains. In future work it would be useful to sequence the PCR product from the strains

that gave false positive reactions, to determine whether, as seems likely, they possess identical primer sites to the *B. intermedia* strains. This analysis might also allow the identification of alternative primer sites that are more specific for *B. intermedia,* and, preferably, which would generate a smaller product size. Leser *et al*. (1997) showed that the PCR could detect 5 x 10^5 cells of *B. intermedia* spiked into pig faeces, and further work should be focused on testing and adapting the PCR for direct detection of *B. intermedia* in faecal specimens from both pigs and chickens.

3.3. Strain Typing using Pulsed Field Gel Electrophoresis

3.3.1. Introduction

To date the genetic relationships between porcine and avian isolates of *B. intermedia* have only been studied using multilocus enzyme electrophoresis (MLEE) (McLaren *et al*., 1997). In this case seven isolates from pigs and 18 from chickens were divided into 17 electrophoretic types (ETs). Five ETs contained multiple chicken isolates from WA, whilst the other 12 ETs contained single isolates, with no ET containing isolates from both pigs and chickens. Overall the species appeared relatively diverse, encompassing a genetic distance of 0.58 (compared to 0.45 for *B. hyodysenteriae*). In the current study pulsed field gel electrophoresis (PFGE) was developed as an alternative means for strain typing, and was used to investigate the genetic diversity and relationships amongst porcine and avian strains of *B. intermedia*.

3.3.2. Materials and methods

The 34 strains of *B. intermedia* used in the preceeding PCR study were used in the current study. The protocol for PFGE was as previously described in our laboratory for *B. pilosicoli* (Atyeo *et al*., 1996). Cells revived from frozen stocks were grown in 300ml volumes until they reached mid log phase, when the cells were harvested by centrifugation (12000 x g, 20 minutes, 4°C, Beckman JA-14 rotor). The cells were resuspended in 100mls of sterile PBS and recentrifuged. This was repeated then the cells were finally resuspended in 0.5mls of 10% sucrose buffer pH 8.0 before being stored at -18°C for later enzyme extraction. This storing buffer prevented the cells from lysing thereby maintained the DNA intact for PFGE analysis.

Frozen cell stocks were thawed and approximately 10^{10} cells were placed in a fresh sterile microfuge tube. The cells were centrifuged at 4500 x g for 5 minutes, and washed twice on ice with chilled sterile PBS. Cells were resuspended in 250µl of sterile PIV buffer, held at room temperature, mixed with an equal volume of 2% low-melt agarose (LMA) in PIV, and pipetted into plug moulds. The agarose was allowed to set at 4°C for 30 minutes, after which the plugs were placed in 2ml of lysis buffer at 37°C for approximately 16 hours with occasional mixing.

Plugs were hardened at 4°C for 20 minutes, placed in 2 mls of ESP solution, and the tubes incubated in a waterbath at 55°C for two hours with occasional mixing. After hardening the plugs at 4°C the two hour incubation was repeated with 2mls of fresh ESP and incubated at 55°C for 16 hours with occasional mixing. The plugs were hardened at 4°C and subjected to six washes with 4mls of sterile TE at 37°C of one hour each. Plugs were then stored in sterile 0.5M EDTA.

Plugs were divided into portions (approximately 5mm x 5mm x 1mm) using a sterile blade. These small plugs were placed into two 1ml changes of TE buffer containing 0.2mM phenylmethylsulfonyl fluoride (PMSF) at 55°C for 30 minutes. Plugs were hardened at 4°C between washes. The plugs were then placed in three 30 minute changes of TE buffer on ice. Restriction digestion of the plugs was done by adding 2µl restriction endonuclease *Mlu*1 (New England Biolabs Inc) and 78µl of 10% restriction buffer and incubating for 16 hours at 37°C. The digested plugs were stored at 4°C prior to loading into 0.5x TBE 1% agarose gels. The plugs were sealed into the agarose gel using 1% LMA in 0.5x TBE, and the gels were chilled at 4°C for 6 hours before being subjected to electrophoresis. A lambda ladder molecular weight marker was included in one lane of each gel.

Gels were loaded onto a contour-clamped homogenous electric field-DR 11 system. Electrophoresis was at 180V for 24 h at 14°C, with the pulse ramped from 1-40 s. Mid-range lambda ladder PFG and wide-range PFG molecular mass markers (New England Biolabs) were included in lanes at both ends of each gel, and were used to normalise isolates. Each strain was tested at least twice. The gels were stained in ethidium bromide solution, then photographed under UV light, and analysed by scanning the photographs (Ofoto 2.0) into the Molecular Analyst program (Bio-Rad). This program created a dendrogram from a matrix of band matching coefficients by the unweighted pair group method of arithmetic averages (UPGMA) clustering fusion strategy.

3.3.3. Results

Six strains were not included in the analysis because of difficulties in preparing and/or digesting their DNA. Clear banding patterns were obtained for the other 28 strains, with each generating between 8 and 15 bands with *Mlu*1. Each strain tested had its own distinct banding pattern. The dendrogram generated from analysis of the patterns showed that the majority of the isolates showed less than 70% genetic similarity to each other. Exceptions included chicken strains 22-5, 22-6 and 22-8 which were isolated from birds on the same farm, which had one and two band differences, and which had an overall genetic similarity of around 90%. The porcine type strain PWS/A^T from England had 82.5% similarity to Swedish porcine strain An520.93, whilst another Swedish porcine strain An983.90 had 78% similarity to Australian porcine strain 889. Other strains were all more distantly related, and the closest similarity between strains from pigs and chickens was 62% for WA strains Wesp1584 isolated from a pig and P3 isolated from a chicken.

The PFGE technique proved to be useful for differentiating between strains of *B. intermedia,* although not all strains yielded DNA suitable for analysis. Generally banding patterns were clear, and since all the strains tested were distinct it was not considered necessary to digest the DNA with additional restriction enzymes. The technique appeared to be more discriminating for differentiation of strains than is MLEE. For example, in a previous MLEE study chicken strains 22-5, 22-6, 22-8, p3 and 3b-1 all were allocated to the same ET (McLaren *et al*., 1997), but in the current study they could all be distinquished, with p3 apparently only showing around 50% similarity with the other strains. A similar situation occurred with chicken strains B52iii, Abb60-9, E2 and A7 which were placed in the same ET, but which here were all distinguished by band differences equated to large genetic differences. The relative merits of the two techniques in terms of estimating genetic differences between strains is uncertain, but results from both techniques suggest that there is considerable overall genetic diversity amongst strains of *B. intermedia.*

As with the dendrogram created by MLEE analysis (McLaren *et al*., 1997), strains from pigs and chickens were distributed throughout the PFGE dendrogram, but there was a tendancy for there to be clusters of more closely related strains isolated from one or other species. The significance of these clusters is uncertain, but, for example, it would be useful to test representative strains from each cluster to determine whether they vary in phenotypic properties, such as their virulence. A few strains from pigs were quite distantly related from the other strains, but they did amplify in the PCR. In future work it would be useful to undertake DNA-DNA reassociation experiments with a range of strains to help to more clearly define the species *B. intermedia*.

None of the strains of *B. intermedia* from poultry and pigs had identical banding patterns, and indeed the most closely related strains from the two species only had a genetic similarity of 62%. Although this did not provide any evidence for the existence of cross-species transmission, this study only examined a small number of strains from diverse geographic localities. Evidence for cross-species transmission could be sought by reciprocal experimental infection of pigs and chicks with avian and porcine strains of *B. intermedia*, and by examining isolates from a limited geographical area, and preferably from farms where both pigs and chicks are kept. Interestingly, Davelaar *et al*. (1986) reported isolating spirochaetes, which were subsequently identified as *B. intermedia* (McLaren *et al.,* 1997), from chickens on a farm where pigs were also kept, and where both species had problems with diarrhoea. Unfortunately no porcine spirochaetes from the farm are available for study. If cross-species transmission is shown to occur this would have important implications for farm management. The availability of a sensitive technique for strain typing by PFGE will facilitate detailed studies designed to help clarify the epidemiology of *B. intermedia* infections.

3.4. Antimicrobial susceptibility testing of intestinal spirochaete isolates

3.4.1. Introduction

There is very litle data available about antimicrobial sensitivities of chicken isolates of intestinal spirochaetes. The only published study tested two US chicken isolates of *B. pilosicoli* and two of *B. alvinipulli* in agar dilution (Trampel *et al.,* 1999). The isolates had high susceptibility to tiamulin, lincomycin and carbadox (drugs used for swine dysentery), but gave highly variable results for a number of other antimicrobials

The purpose of the present study was investigate a range of Australian and some overseas chicken isolates of the various *Brachyspira* species that infect poultry for their sensitivity to seven antimicrobial drugs that have been used to treat intestinal spirochaete infections in pigs. The overall aim was to determine the likely effectiveness of antimicrobial drugs for the control of AIS in the field. The seven antimicrobials were tested on 80 chicken isolates using the standard agar dilution technique.

3.4.2. Materials and methods

Source of isolates

A total of 80 intestinal spirochaete strains were tested. The Australian isolates were collected in the current and in previous studies (McLaren *et al.,* 1996; Stephens and Hampson, 1999). These included 16 isolates of *B. pilosicoli* from Australian poultry flocks (14 from Queensland, one from Western Australia and one from Tasmania), as well as two isolates from the Netherlands and four from the USA. These isolates are listed in Table 4. Another 57 isolates of non-*B. pilosicoli* species from Australian poultry flocks were examined. These consisted of 28 isolates from Queensland (two isolates of *B. innocens*, 10 isolates of *B. intermedia,* two isolates of "*B. pulli*" and 16 isolates of *B. murdochii*), and 10 isolates of *B. intermedia* from Western Australian flocks. One isolate of *B. intermedia* from the Netherlands was also examined.

Culture conditions and cell counts

Pure cultures of isolates were taken from -70°C storage and inoculated onto blood agar plates (TSA with 5% defibrinated ovine blood). These were incubated in an atmosphere of 94% N_2 6%O_2 at 37°C for 5 days. After this period the cells were gently resuspended in 1 ml of sterile PBS using a flame sterilised L-shaped glass rod. The resuspended cells were counted using a haemocytometer before drop inoculating 10^5 cells (volumes ranged from 2 to 20µl depending on the density of growth) onto the agar diffusion sensitivity plates.

Antimicrobial stock solutions

Antimicrobial stock solutions were made by mixing the following antimicrobial powders with sterile distilled water (source and activity in parentheses): tiamulin hydrogen fumerate (Novartis Animal Health) (99.5%), lincomycin hydrochloride (Sigma) (808 units/mg), tylosin tartrate (Sigma; 906µg/mg), metronidazole (Sigma; 100%), tetracycline hydrochloride (Progen Industries; 950µg/mg), neomycin sulphate (Sigma; 734µg/mg), ampicillin (Sigma; 100%). Stock solutions were stored at 4°C for less than 24 hours before use.

Agar Dilution Test and Minimum Inhibitory Concentration (MIC) Estimation

Antimicrobial susceptibility plates were made using TSA supplemented with 5% defibrinated ovine blood. Four concentrations of each antimicrobial were tested, and the appropriate amount of each stock solution was added to the agar before pouring the plates. Table 3 shows the concentrations used for this experiment. The antimicrobial sensitivity plates and control plates containing TSA with 5% ovine blood were dried for 15 minutes at 37°C before inoculation with the measured amount of test organisms. The inoculum was allowed to dry before incubating in an atmosphere of 94% N_2 and 6% CO_2 at 37°C. Isolates were considered resistant to the antimicrobial concentration if zones of haemolysis were observed around inoculated spirochaetes after five days. Plate cultures were checked for growth of spirochaetes and purity by direct microscopic examination to confirm the endpoint. Since serial dilutions were not performed the MIC values were recorded as being in the range between the highest sensitive concentration and the lowest resistant concentration.

Table 3: The concentrations used in the agar dilution antimicrobial sensitivity test.

antimicrobial agent	concentrations tested (µg/ml)
tiamulin	0.1, 1, 5, 10
lincomycin	1, 10, 50, 100
tylosin	5, 20, 50, 100
metronidazole	0.1, 1, 10, 20
tetracycline	1, 5, 10, 20
neomycin	10, 50, 100, 200
ampicillin	1, 10, 50, 100

3.4.3. Results and discussion

The MIC results for the *B. pilosicoli* isolates are presented in Table 4, and the overall results for all the isolates are shown in Figure 1. Unfortunately, as yet there are no standards approved or recommended for antimicrobial susceptibility testing of *Brachyspira* spp. However, antimicrobial susceptibility testing of *B. hyodysenteriae* isolates is done mainly by agar dilution (Rønne and Szancer, 1990), and it was appropriate to use the same technique for the other *Brachyspira* spp. tested here. Breakpoints have not been established for all antimicrobials or for *Brachyspira* species other than *B. hyodysenteriae*. For this species, however, the proposed resistance breakpoint for tiamulin is 4 µg/ml, and for lincomycin it is >36 µg/ml (Rønne and Szancer, 1990).

Overall sensitivity to the antimicrobial drugs tested was generally high, although the different spirochaete species tended to show different patterns of sensitivity. Three isolates of *B. intermedia* (QAW 1, QAW 2, QAW 15) had MIC values of between 1 and 5µg/ml to tiamulin, whilst all the other isolates tested had lower MIC values. In this case it would have been useful to have included a greater range of concentrations of the drug, since the breakpoint is probably in this region. Some isolates of *B. pilosicoli*, *B. intermedia* and *B. pulli* recorded higher MIC values against lincomycin (10-50µg/ml) and tylosin (>100µg/ml) than the other species tested, and in general this tendency for resistance, particularly with tylosin, would not make these drugs of first choice for control of AIS, in the absence of sensitivity testing. All species exhibited similar high susceptibilities to metronidazole. *B. pilosicoli* isolate 42167 from the USA was the most resistant to tetracycline, with an MIC value of 10-20µg/ml, probably indicating resistance, whilst other species had similar MIC values. Most of the isolates of *B. pulli* and *B. pilosicoli* were found to be less susceptible to neomycin than other species, exhibiting MIC values of 50-100µg/ml. Half of the isolates of *B. pulli* and *B. innocens* tested had MIC values of 10-50µg/ml against ampicillin, the other half and almost all of the isolates *of B. intermedia* and *B. murdochii* were more susceptible to this antimicrobial (MIC <1).

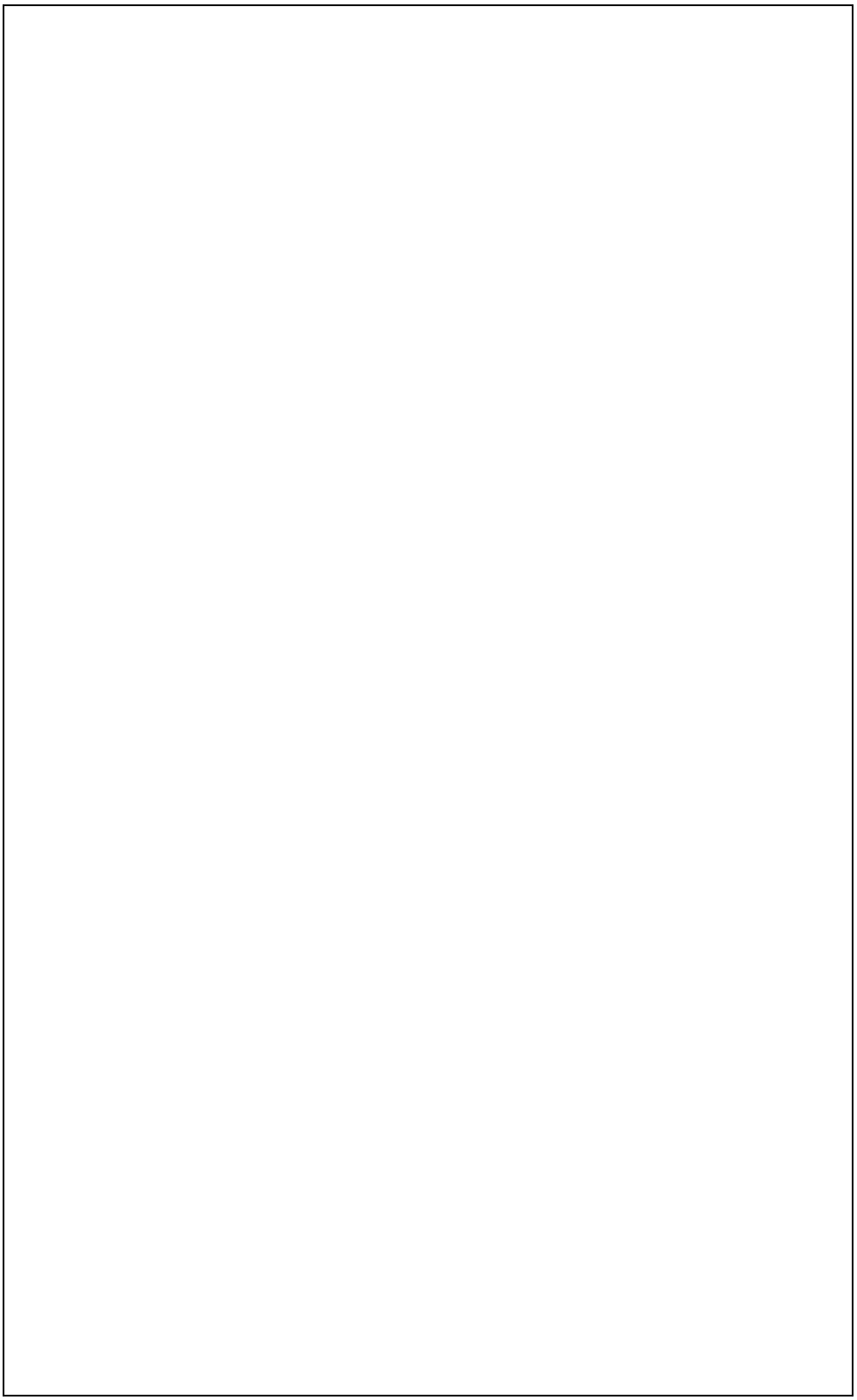
Overall, considering the two pathogenic species examined, *B. intermedia* was most sensitive of all the species to ampicillin, and most resistant to tiamulin and tylosin. *B. pilosicoli* was most sensitive to tiamulin, and most resistant to ampicillin and neomycin. The different results for the two species with tiamulin could be problematic for treatment, and again this indicates the importance of isolating and identifying the spirochaetes, and testing their MIC values, before treating flocks with AIS. In the absence of availability of such testing, the current results provide some general guide as to prefered treatment.

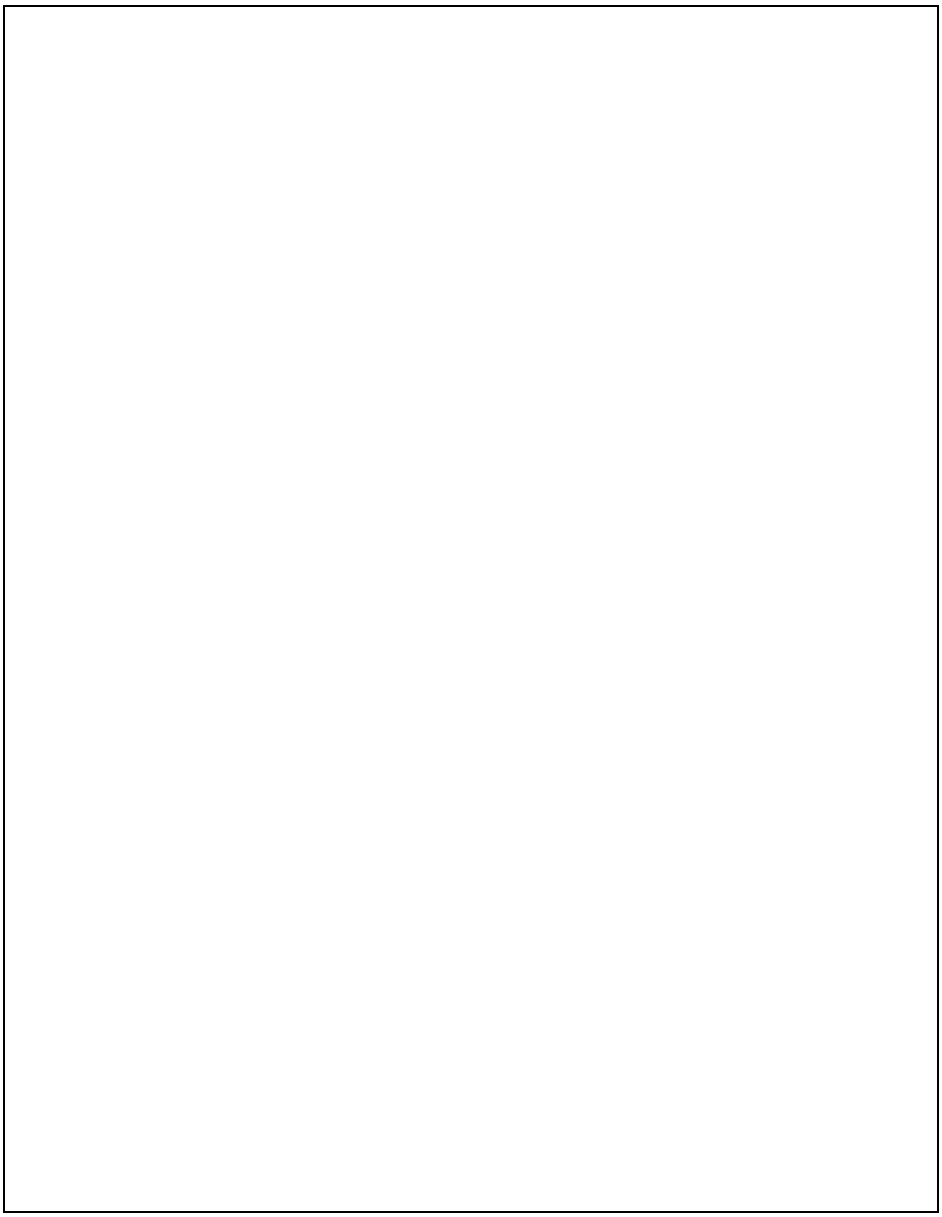
Table 4: MIC values of isolates of *B. pilosicoli* obtained from poultry to seven antimicrobials.

Origin	isolate name[a]	lincomycin	ampicillin	tiamulin	tylosin	tetracycline	metronidazole	neomycin
Qld. Aust.	QAP1	1-10	>100	<0.1	50-100	1-5	0.1-1	100-200
Qld. Aust.	QAP3	10-50	<1	<0.1	>100	<1	0.1-1	100-200
Qld. Aust.	QAP4	1-10	10-50	<0.1	>100	<1	0.1-1	50-100
Qld. Aust.	QAP5	<1	10-50	<0.1	<5	<1	<0.1	100-200
Qld. Aust.	QAP6	1-10	>100	<0.1	5-20	1-5	0.1-1	100-200
Qld. Aust.	QAP7	1-10	10-50	<0.1	<5	<1	<0.1	50-100
Qld. Aust.	QAP10	<1	10-50	<0.1	<5	<1	0.1-1	100-200
Qld. Aust.	QAP8a	<1	<1	<0.1	5-20	<1	0.1-1	100-200
Qld. Aust.	QAP12	<1	<1	<0.1	<5	<1	0.1-1	100-200
Qld. Aust.	QAP14	<1	<1	<0.1	<5	<1	0.1-1	100-200
Qld. Aust.	QAP15	<1	<1	<0.1	<5	<1	0.1-1	100-200
Qld. Aust.	QAP16	10-50	10-50	<0.1	>100	<1	0.1-1	100-200
Netherlands	13316	10-50	<1	<0.1	>100	<1	0.1-1	100-200
West Aust.	1772	<1	1-10	<0.1	50-100	1-5	0.1-1	100-200
Iowa, USA	42167	10-50	1-10	0.1-1	>100	10-20	<0.1	50-100
Iowa, USA	R4	<1	1-10	0.1-1	50-100	1-5	<0.1	50-100
Nebraska, USA	92.S76	1-10	<1	<0.1	50-100	1-5	0.1-1	100-200
Netherlands	4742	10-50	<1	0.1-1	>100	<1	0.1-1	100-200
Qld. Aust.	Qu1	1-10	<1	0.1-1	5-20	1-5	0.1-1	50-100

[a]Note: origin of isolate R4 rhea (*Rhea americana*), isolate 92.S76 chiloe widgeon (*Anas sibilatrix*), and all other isolates from chickens (*Gallus domesticus)*.

Fig 1. Comparisons of antimicrobial susceptibilities of *Brachyspira* spp. isolated from poultry to seven antimicrobial agents





3.5. Multilocus Enzyme Electrophoresis

3.5.1. Introduction

Multilocus enzyme electrophoresis (MLEE) has been widely used for the study of intestinal spirochaetes, and, for example was used in our laboratory to define the main species of intestinal spirochaete that infect poultry (McLaren *et al.*, 1997). It is currently the best global technique for identifying all species, and also provides information about relatedness between species and strains of the bacteria. The current MLEE study was undertaken to help support the species identification of chicken isolates, and in particular to aid in our assessment of sensitivities and specificities of the

PCR techniques being used and developed in our laboratory. The study also provided additional information about the genetic relatedness and diversity of intestinal spirochaete isolates from Australian chickens, and compared these with non-Australian isolates.

3.5.2. Materials and methods

Source of isolates

A total of 62 intestinal spirochaete isolates were examined by MLEE. These comprised 54 isolates from Australian poultry flocks obtained during this study, together with two other Australian isolates (1772 and Qu1) from a previous study (McLaren, *et al.,*1997), and six isolates from poultry originating in the USA and the Netherlands (Table 5).

Preparation of enzymes

Cells revived from frozen stocks were grown into 300ml volumes in the manner described in Section 2.1.2. Once the culture had reached mid log phase, cells were harvested by centrifugation (12 000 x g, 20 minutes, 4°C, Beckman JA-14 rotor). The cells were resuspended in 100mls of sterile PBS and recentrifuged. This was repeated then the cells were finally resuspended in 1 ml of sterile distilled water before being stored at -20°C for later enzyme extraction.

Centrifuged cells were thawed at 37°C and immediately placed on ice. Cells were lysed while on ice by sonication using a Sonicator XL2015 ultrasonic liquid processor (Heat Systems, New York, USA) and then centrifuged (15000 x g, 10 minutes, 4°C). The supernatant was recovered and stored at -70°C until used.

The allelic profiles of the same 15 constitutive enzyme loci used in our laboratory by Lee were examined (Lee *et al.*, 1993). These enzymes were: acid phosphatase (ACP), alcohol dehydrogenase (ADH), alkaline phosphatase (ALP), arginine phosphokinase (APK), esterase (EST), fructose 1,6 diphosphate (FDP), glutamate dehydrogenase (GDH), guanine deaminase (GDA), hexokinase (HEX), L-leucyl-glycyl-glycine peptidase (LGG), mannose phosphate isomerase (MPI), nucleoside phosphorylase (NP), phosphoglucomutase (PGM), phosphoglycose isomerase (PGI) and superoxide dismutase (SOD). The various enzymes were run in four different buffer systems: ACP, ADH, HEX and NP were assayed using a Tris malate (pH 7.4) buffer system; ALP, PGI, GDA and MPI were run in a phosphate (pH 7.0) buffer system; EST, FDP, LGG, PGM and SOD were assayed in a discontinuous lithium hydroxide buffer system; APK and GDH were assayed in a discontinuous Poulik buffer system.

The cell supernatant containing enzymes for each isolate was mixed in a 3:1 ratio with enzyme extractant buffer (2% sucrose, 2% mercaptoethanol, 2% bromophenol blue), loaded onto Whatman

No 3 filter wicks (0.4cm x 0.8cm) and slotted into the bottom of a 12.8% horizontal starch gel containing the corresponding gel buffer.

The variation in the electrophoretic mobility of each enzyme was interpreted as the products of different alleles at the locus coding for that enzyme. Distinctive mobility variants were numbered in order of decreasing rate of anodal migration according to the system previously adopted by Lee *et al.* (1993). Isolates that did not show activity for some of the enzymes were assigned a null allele (zero). For pairwise comparison, the null alleles were not included in the analysis and were assigned the value -1, since the factors causing the enzyme not to appear may not have been uniform for all the isolates. Gel runs were repeated up to four times to ensure correct allele designation.

Isolates with identical enzymatic profiles at all loci were grouped into an electrophoretic type (ET). ETs obtained for the new isolates were directly compared with standards obtained in previous MLEE studies (Lee *et al.,* 1993).

Genetic diversity (*h*), a measure of the amount of allelic variation at each enzyme locus, was calculated for the number of ETs as $h = (1-\sum pi^2)(n/n-1)$ where *pi* is the frequency of the indicated allele and *n* the number of ETs. Genetic distance between ETs was calculated by pairwise comparison as the proportions of loci at which dissimilar alleles occurred. A phenogram was generated to illustrate the genetic relationships between newly identified ETs using the unweighted paired group method of arithmetic averages clustering fusion strategy. The results from the current study were overlayed on results previously obtained for avian intestinal spirochaetes by McLaren *et al* .(1997). The computer program Phentree was used to generate the phenogram data from pairwise distance matrixes, using the programme Phylip to generate a phenogram.

3.5.3. Results and discussion

The MLEE grouping, PCR results, and clinical symptoms exhibited by poultry flocks from which the isolates originated are presented in Table 5. The MLEE grouping can be seen in Figure 2, in which is also shown the grouping of other known isolates as described in the study of McLaren *et al.* (1997). The number of Australian isolates of each species and their ET distribution is shown in Table 6. These isolates came from 15 layer and broiler breeder flocks, and more than one spirochaete species was isolated from five of these flocks.

For *B. pilosicoli* isolates there was a perfect correlation between MLEE grouping (m) and PCR results, with 16 isolates from five flocks divided into 6 ETs. For the 13 *B. intermedia* isolates, these were defined by their location in group g on the phenogram. Of these 13, only four were positive in the *nox* PCR, but all were positive in the new 23S rRNA PCR. The isolates were from six Australian

flocks, and belonged to 11 ETs. As with *B. pilosicoli*, more than one ET was found on the same farm. Hence multiple strains of these species may be present in certain flocks, and this may make treatment more difficult if they have different drug sensitivities or vary in some other important properties. The *nox* PCR for the combined species *B. innocens*/*B. murdochii* was positive in nine cases. Seven of these isolates belonged to MLEE group d, identified as *B. murdochii*, one was in group b (a new unnamed group), and one in group a (also new and unnamed). Interestingly ten other isolates in group d (*B. murdochii*) were negative in the PCR, as were three in group e (*B. innocens)*. These results indicate that the *B. murdochii*/*B. innocens nox* PCR is specific, but has poor sensitivity of detection for these two species. This is probably not a major problem, since neither species is considered pathogenic in poultry. However, it is worth noting (Table 6) that these isolates all came from flocks with wet litter or production problems, and it is still necessary to clarify their pathogenic potential by testing them under experimental conditions.

Overall, eight new genetic groups of intestinal spirochaetes were identified by MLEE (groups a, b, c, h, i, j, k and l on the phenogram). Further work is required to clarify whether these groups represent new species, and whether they have any significance in relation to disease production. As in our previous study (McLaren *et al.,* 1997), no isolates of *B. alvinipulli* were identified amongst the Australian isolates. To date *B. alvinipulli* has only been isolated from a single diseased flock in the USA (Swayne *et al*., 1995).

Table 5: Isolate name, PCR result, MLEE grouping, age of bird sampled, name of flock and the reason for submission of the samples of 55 isolates of *Brachyspira* spp. from poultry.

isolate name	PCR[a]	MLEE group	Age of bird (weeks)	Flock name[b]	Reason for sample submission
Qu1	*B. pilosicoli*	m	NA	N	NA
QAP1	*B. pilosicoli*	m	NA	M	Survey
QAP3	*B. pilosicoli*	m	32	B	Severe pericarditis, diarrhoea
QAP4	*B. pilosicoli*	m	50	C	depressed/lethargic/off lay
QAP5	*B. pilosicoli*	m	NA	M	survey
QAP6	*B. pilosicoli*	m	NA	M	survey
QAP9	*B. pilosicoli*	m	21	P	NA
QAP7	*B. pilosicoli*	m	NA	M	survey
QAP11	*B. pilosicoli*	m	NA	**M**	survey
QAP8a	*B. pilosicoli*	m	NA	**A**	Wet litter in 20% of flock
QAP10	*B. pilosicoli*	m	NA	M	survey
QAP12	*B. pilosicoli*	m	NA	**A**	Wet litter
QAP13	*B. pilosicoli*	m	NA	**A**	Wet litter
QAP14	*B. pilosicoli*	m	NA	**A**	Wet litter
QAP15	*B. pilosicoli*	m	NA	**A**	Wet litter
QAP16	*B. pilosicoli*	m	NA	**A**	Wet litter
QAP17	negative	l	36	D	NA
QAW5	*B. inno/murd*	b	NA	J	NA
QAW37	negative	j	NA	D	NA
QAW28	negative	e	NA	D	NA
QAW33	negative	e	NA	G	NA
QAW38	negative	e	NA	O	Diarrhoea
QAW34	negative	f	74	H	NA
QAW36	negative	h	43	**L**	Diarrhoea/ depressed egg production/ lethargy
QAW7	negative	h	NA	**A**	Wet litter
QAW1	negative	g	62	C	obvious illness
QAW2	*B. intermedia*	g	62	C	obvious illness
QAW3	negative	g	62	C	obvious illness
QAW4	*B. intermedia*	g	21	K	Poor growth rate/ death
QAW12	negative	g	32	B	Diarrhoea/death

QAW15	*B. intermedia*	g	32	B	Diarrhoea/death
QAW17	negative	g	32	B	Diarrhoea/death
QAW16	negative	g	NA	B	Diarrhoea/death
QAW18	negative	g	NA	M	Survey
QAW20	*B. intermedia*	g	41	**I**	NA
QAW22	negative	g	NA	M	Survey
QAW40	negative	g	15	E	Death
QAW32	negative	g	NA	E	Tumors in liver and ovary
QAW14	*B. inno/murd*	a	NA	**A**	Wet litter
QAW6	*B. inno/murd*	d	NA	**A**	Wet litter
QAW8	*B. inno/murd*	d	NA	**A**	Wet litter
QAW9	*B. inno/murd*	d	NA	**A**	Wet litter
QAW10	*B. inno/murd*	d	NA	**A**	Wet litter
QAW11	negative	d	NA	**A**	Wet litter
QAW19	*B. inno/murd*	d	25	**A**	Extreme wasting of birds/ frothy caecal contents at PM
QAW21	negative	d	NA	**A**	Wet litter
QAW23	negative	d	NA	**A**	Wet litter
QAW24	*B. inno/murd*	d	NA	**A**	Wet litter
QAW25	negative	d	40	**A**	Poor flock production/ diarrhoea
QAW26	negative	d	NA	**A**	Wet litter
QAW27	negative	d	NA	**A**	Wet litter
QAW29	negative	d	NA	D	NA
QAW30	negative	d	NA	**A**	Wet litter
QAW31	negative	d	NA	**A**	Wet litter
QAW35	negative	d	NA	**A**	Wet litter

NA, data was not available

[a] PCR for *B. pilosicoli* based on 16S rRNA gene amplification. Other PCRs based on *nox* gene amplification.
[b] where isolates in bold designates the flock is a broiler breeder flock, otherwise from layer flock

Table 6: The number of isolates and number of ETs by MLEE of *B. pilosicoli*, *B. intermedia*, *B. murdochii, "B. pulli"*, *B. innocens* and other unnamed WBHIS into which 55 isolates from 15 Australian poultry flocks were grouped.

Species	No ETs by MLEE	No of isolates	Signs of disease in affected flocks	Flocks colonised[a]
B. pilosicoli	6	16	wet litter, diarrhoea, lethargy	**A**, B, C, **M**, N
B. intermedia	11	13	poor growth, diarrhoea, death, tumours	B, C, E, I, K, **M**
B. innocens	2	2	wet litter, diarrhoea	D, G
B. murdochii	14	16	wet litter, poor production	A, D
"B. pulli"	2	2	wet litter, poor production	H, O
Other groups	6	6	diarrhoea, wet litter, poor egg production	**A**, D, J, **L**

[a] Where bold designates the flock is broiler breeder and normal designates a layer flock

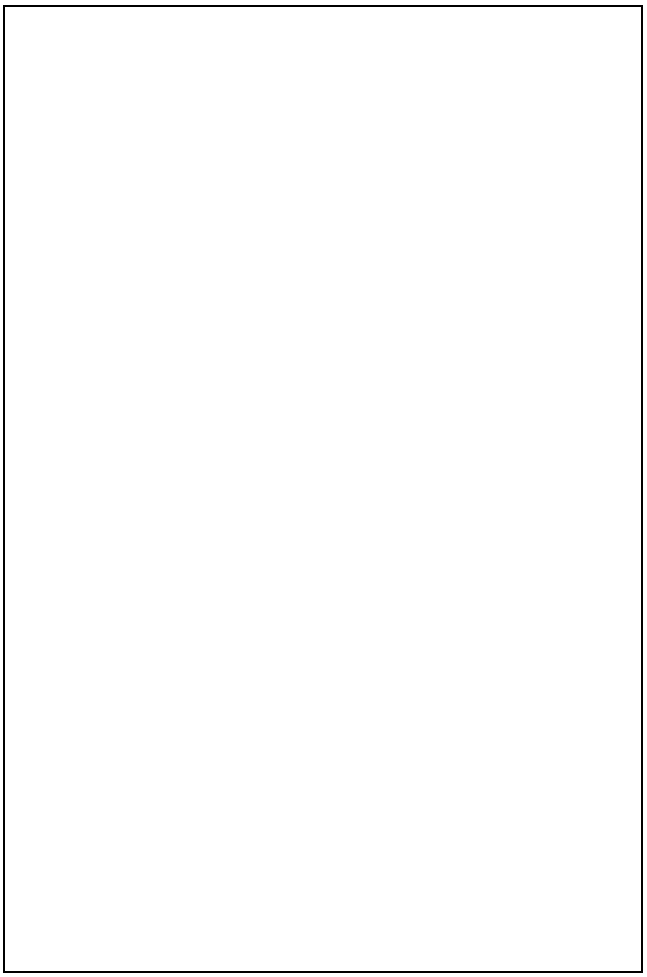


Fig 3: Phenogram of genetic distance among 82 ETs containing intestinal spirochaetes isolated from chickens.

Groups a, b, c, h, i, j, k and l are newly identified unnamed groups. Isolate names written on the phenogram correspond to those in McLaren *et al* (1997). .

4. Experimental infection studies

4.1 Experimental infection studies in layer hens

The studies in layer hens described in this section were all conducted at Murdoch University, and were undertaken with the approval of the Murdoch University Animal Ethics Committee. They consisted of three experiments, which were intended to investigate the use of tiamulin and lincomycin for the control of AIS caused by *B. intermedia* and *B. pilosicoli*, as well as to evaluate the potential of dietary enzymes to help control intestinal spirochaete infections. During the course of the experiments, due to unforseen circumstances, an opportunity also became available to examine the interaction of zinc bacitracin on AIS.

4.1.1. Influence of in-feed zinc bacitracin and tiamulin treatment on experimental avian intestinal spirochaetosis caused by *Brachyspira intermedia*

Summary

Thirty individually caged layer hens were inoculated with *Brachyspira intermedia,* and 20 control birds remained unchallenged. Birds received a diet containing 100 ppm zinc bacitracin (ZnB), and were monitored for ten weeks. *B. intermedia* was recovered sporadically from five of the inoculated birds, and there were no significant effects on body weight, faecal water or egg production. ZnB was presumed to be inhibiting spirochaete growth, and when removed from the diet, 18 of the 30 inoculated birds rapidly became culture positive. After 4 weeks, 10 of the 30 infected birds were treated with tiamulin at 25mg/kg for 5 days, and 10 were returned to the diet containing ZnB. Birds receiving tiamulin became spirochaete negative, and maintained their egg production, but reinfection occurred. The other 20 infected birds had a significant drop in egg production, but those receiving ZnB showed a reduced colonisation by *B. intermedia* after three weeks.

Introduction

The purpose of the present study was to evaluate the therapeutic effects of a short course of tiamulin (a pleuromutilin antimicrobial) in layers held in controlled conditions and experimentally infected with a single pathogenic strain of *B. intermedia.* Inadvertently, the diet initially used in the experiment contained 100 ppm of zinc bacitracin (ZnB: a polypeptide antimicrobial), and this occurrence provided an opportunity also to examine the efficacy of ZnB against colonisation with the spirochaete.

Materials and methods

Experimental birds

Fifty 18-week-old ISA-Brown layer hens were purchased from a commercial breeder. The birds were housed in a climate controlled (21-23^{o}C) facility, and randomly allocated to individual cages with mesh floors set in banks of 10 cages. Thirty of the birds that were to be infected were placed in one room, and 20 uninfected control birds in an adjacent room. The birds were subjected to 12 hours artificial light each day.

Experimental infection

Brachyspira (*Serpulina*) *intermedia*, Western Australian chicken strain HB60, was obtained as a frozen stock culture from the collection held by the Reference Centre for Intestinal Spirochaetes at Murdoch University. This was thawed and grown in Kunkle's anaerobic broth medium (Kunkle *et al*., 1986). Thirty birds were orally inoculated via a crop tube with two mL of an actively growing culture on three consecutive days per week for three weeks (ie nine inoculations), starting one week after the birds were obtained. The broth contained approximately 10^8 bacterial cells per mL. The 20 uninfected control birds in the other room were inoculated with sterile broth.

Diet

The birds were fed *ad libitum* on a commercial, vegetable-based layer diet provided by the commercial producer who supplied the birds. Inadvertently, the diet fed to the birds initially contained ZnB at 100 ppm. The ZnB was removed after ten weeks, when the birds were 28 weeks old, all birds then being given the same diet free of ZnB. After four weeks, when the birds were 32 weeks of age, 100 ppm ZnB was re-introduced into the diet of half (10) of the control birds, and 10 of the 30 experimentally infected birds. All birds had constant access to water. The birds were killed five weeks later, when they were 37 weeks of age.

Treatment with tiamulin

Thirteen weeks after the start of the experiment, when the birds were 32 weeks of age, 10 of the 20 infected birds which remained on the ZnB-free diet were orally dosed daily on five consecutive days by crop tube with an approximate 2ml solution of tiamulin (Novartis Animal Health, Sydney, Australia) dissolved in sterile water, at a rate of 25 mg tiamulin per kg bodyweight per day.

Monitoring for spirochaetes in faeces

At the end of each week, aluminium foil was placed under the cage of each bird, and after one hour individual faecal samples were collected into sterile tubes. These were cultured for

spirochaetes on selective Trypticase Soy Agar (BBL, Cockeysville, MD) supplemented with 5% defibrinated ovine blood, 400 µg/ml spectinomycin and 25 µg/ml each of colistin and vancomycin (Jenkinson and Wingar, 1981). Plates were incubated in an anaerobic environment generated by Gaspak *Plus* sachets (BBL), and growth was examined by phase contrast microscopy after 5 and 10 days. Total bacterial growth on the primary plate was then scraped off with a sterile glass slide, and an aliquot subjected to a polymerase chain reaction (PCR) specific for the 23S rRNA gene of *B. intermedia*, as previously described (Suriyaarachchi *et al*., 2000).

Faecal moisture content

The remainder of the faecal samples were weighed, then dried to constant weight in a hot air oven to determine their faecal moisture content.

Egg production

Egg numbers and egg weights for each bird in each group were recorded daily, and accumulated to provide weekly egg mass output.

Body weights

The birds were weighed weekly throughout the experiment.

Post-mortem examination

When the birds were 37 weeks of age they were killed by cervical dislocation, and subjected to post-mortem examination. The caeca and colon were opened to look for evidence of gross changes, sections of one caecum placed in 10% buffered formalin for subsequent histological examination, and swabs were taken from the other caecum for spirochaete culture. The fixed tissue was processed through to paraffin blocks, cut at 4µm and stained with haematoxylin and eosin.

Statistical analysis

For the first part of the experiment, up until the time the birds were divided into five treatment groups at 32 weeks of age, comparisons were made between the 30 infected birds and the 20 uninfected birds. After then, comparisons were made between all five groups. Weekly and overall group bird weights, faecal moisture content and total egg numbers and egg mass production (egg numbers multiplied by egg weights) were compared using one-way analysis of variance. Means were compared using Fisher's protected least significant difference method, and significance was accepted at the 0.5% level.

Results

Bird weights

Birds in all groups gained weight throughout the course of the experiment, and there were no significant effects of infection or treatment on group body weights at any time.

Faecal moisture

No statistically significant differences were found in the faecal moisture content of the birds in the various groups at any time during the experiment.

Detection of spirochaetes

All faecal cultures carried out prior to inoculation of the birds were negative, and all faecal cultures of the twenty control birds were negative throughout the trial. In the period following inoculation, up until the time the ZnB was removed from the diet when the birds were 28 weeks of age, a maximum of five of the thirty experimentally inoculated birds were culture positive (Table 7). In each week, however, additional birds were shown to be colonised by *B. intermedia* by using PCR, with a maximum of 16 birds being PCR positive at 26 weeks of age. The number of birds that were culture positive increased markedly (to 18) immediately following removal of ZnB from the diet (Table 7). Twenty five of the 30 birds were PCR positive at 29 weeks of age.

In the period from 32 weeks of age until the end of the experiment, between five and seven of the 10 infected birds that remained on the diet without ZnB were culture positive each week (Table 8). In contrast, only four of the birds put back on the ZnB were culture positive after one week, and in weeks 35 and 36 none were culture or PCR positive, although the caeca of three were culture positive at post-mortem examination at 37 weeks of age (Table 8). One week after treatment with tiamulin, all 10 birds were negative when cultured for spirochaetes, although one was PCR positive (Table 8). The number of colonised birds increased slowly over the next five weeks, until nine out of the 10 treated birds were positive at post-mortem examination at 37 weeks of age.

Table 7: Faecal excretion of *B. intermedia* by 30 experimentally infected birds, as detected by direct culture and by PCR of total bacterial growth from the primary isolation plate

Age of bird (weeks)	No. of birds culture positive	No of birds PCR positive
19	0	0
20	0	0
21	0	2
22	0	0
23	1	3
24	3	9
25	1	3
26	4	16
27	5	6
28[a]	3	3
29	18	25
30	13	13
31	18	20

[a]Zinc bacitracin removed from the diet at 28 weeks of age.

Table 8: Faecal excretion of *B. intermedia* by 3 groups of 10 experimentally infected birds, as detected by direct culture and by PCR of total bacterial growth from the primary isolation plate

Age of birds	No ZnB		ZnB[a]		Tiamulin, no ZnB[b]	
(weeks)	Culture	PCR	Culture	PCR	Culture	PCR
32	7	8	4	5	0	1
33	7	7	3	3	1	1
34	6	7	3	3	3	3
35	5	5	0	0	4	4
36	7	7	0	0	6	8
37[d]	5	7	3	4	9	9

[a]Zinc bacitracin in the diet throughout weeks 32-37

[b]Tiamulin given in the water for 5 days at the end of week 31

[d]Results from caecum at post-mortem examination

Egg numbers and total egg production

No significant differences were found in egg numbers or total egg mass output (egg numbers multiplied by egg weights) between the inoculated (n=30) and control (n=20) birds up until the birds were divided into five groups at 32 weeks of age. At 34 weeks of age, egg numbers and egg mass output were significantly less in the infected birds without ZnB than in the birds treated with tiamulin (Tables 9 and 10). No other differences were significant. At 35 weeks, egg mass output was significantly reduced in the two infected groups not receiving tiamulin. At 36 weeks, the infected group without ZnB had significantly worse egg mass output than all the other groups, except the infected group receiving ZnB. At 37 weeks, the infected group receiving ZnB and the control group receiving ZnB had significantly lower egg numbers than the other groups. The total egg mass output of the control group receiving ZnB was not reduced however, due to the larger size and greater weight of the eggs produced (Table 10).

Post-mortem findings

The caecal size and contents varied considerably between birds, with some caeca being small and empty, and others being large and full of contents varying in consistency from firm, through sticky, to watery and frothy. There were no consistent group effects on these findings, although only three of the control birds had contents that were not firm. The mucosal surface of most caeca were grossly normal, but several were slightly congested, and seven had small areas of petechial haemorrhage. Five of the latter were in infected birds not receiving ZnB, and two were in birds that had received tiamulin. Histological examination showed mild inflammatory changes in some of the samples from all groups, with congestion and slightly increased numbers of mixed inflammatory cells in the lamina propria. No epithelial erosion was observed, and the enterocytes were generally columnar. Large numbers of spirochaetes were observed in the lumen of the crypts and overlying the lumenal epithelium in the birds from which spirochaetes were isolated.

Table 9: Weekly group mean egg numbers following allocation of birds to five groups

Age of birds (weeks)	Control (no ZnB[a])	Control (ZnB)	Infected (tiamulin, no ZnB)	Infected (no ZnB)	Infected (ZnB)	*P*- value
32	5.3	5.8	5.2	5.2	5.6	0.3655
33	6.4	6.5	6.0	6.5	5.8	0.3787
34	6.0	6.0	6.1[b]	5.2[a]	5.4	0.1532
35	7.1[a]	6.5[ab]	7.4[a]	5.6[b]	5.7[b]	0.0035
36	6.6[a]	5.9[ab]	6.6[a]	5.1[b]	5.4[b]	0.0205
37	6.6[a]	4.5[b]	6.5[a]	5.8[ab]	4.6[b]	<0.0001

ZnB, zinc bacitracin

Within rows, group means with a different superscript differ at the 5% level of significance

Table 10: Weekly group mean egg mass output (number of eggs multiplied by weight) following allocation of birds to five groups

Age of birds (weeks)	Control (no ZnB)	Control (ZnB)	Infected (tiamulin, no ZnB)	Infected (no ZnB)	Infected (ZnB)	*P*-value
32	308.7	353.9	323.0	314.7	344.6	0.1571
33	376.5	396.7	371.2	404.6	360.6	0.4929
34	355.5	364.9	383.8[b]	324.3[a]	337.0	0.2262
35	423.2[ac]	399.7[a]	471.7[c]	340.3[b]	353.4[b]	0.0021
36	393.1[ac]	356.5[c]	423.0[c]	313.5[b]	332.0	0.0141
37	400.0	385.0	417.5	344.7	280.7[a]	<0.0001

ZnB, zinc bacitracin

Within rows, group means with a different superscript differ at the 5% level of significance

Discussion

The *B. intermedia* strain used in this experiment was isolated from a Western Australian chicken in a flock with wet litter and production problems (McLaren *et al.,* 1996). The strain has previously been used to experimentally infect layer hens, causing increased faecal moisture and reduced egg production (Hampson and McLaren, 1999). In the current experiment, colonisation rates with *B. intermedia* were low following experimental inoculation of 30 birds, despite nine individual inoculations with the strain. Consistent with this, no significant effects of inoculation on bird weights, egg production or faecal water content were seen during this period. The use of PCR on growth from the primary plate considerably increased the number of colonised birds that were detected, and this result helps to emphasise the usefulness of this technique to supplement direct culture. The samples that were PCR positive but culture negative presumably had very low numbers of organisms present.

Whilst seeking a reason for the unexpected initial low rate of colonisation and lack of effect on production, it was discovered that the diet inadvertently had been supplied containing 100 ppm ZnB. Within one week of removing the ZnB from the diet, a proliferation of *B. intermedia* was detected amongst the previously inoculated birds, and this colonisation persisted, with between half and two thirds of the birds maintained on the ZnB-free diet being culture positive each week through to the end of the experiment. Interestingly, the removal of the ZnB did not alter faecal water content in either the control or infected birds. The assumption made from these observations was that the ZnB was inhibiting growth of the spirochaete, and that the ZnB itself had no other obvious effect on production. The inhibition of spirochaete growth by the ZnB could have occurred either via direct antibacterial effects on the spirochaete, or possibly through an influence on other components of the intestinal flora, which in turn interact with the spirochaete.

Although spirochaetes proliferated following the withdrawal of ZnB, there was no effect on faecal moisture content throughout the rest of the experiment, nor any immediate effect on egg production. However, seven to nine weeks following the withdrawal (two to five weeks following the allocation of the birds to five groups at 32 weeks of age), the group of 10 infected birds still on the diet without ZnB started to produce significantly fewer and lighter eggs (tables 9 and 10). The infected birds reintroduced to ZnB also had significantly reduced egg numbers around this time. This depression in egg production followed, but lagged well behind, the increase in numbers of birds colonised by *B. intermedia*. The loss of egg production was consistent with the reported pathological effects of *B. intermedia* (Hampson and McLaren, 1999), but the experiment was not of sufficient duration to determine the extent of the effect. Furthermore, potential egg production losses probably were minimised by the good husbandry, lack of crowding, and absence of other pathogens in the experimental birds, compared to those kept under commercial conditions.

Treatment with tiamulin resulted in an immediate almost complete clearing of *B. intermedia* infection, but colonisation rates started to increase again after two weeks, and all but one of the birds were colonised at slaughter. Despite the recolonisation, treatment with tiamulin maintained egg production at levels similar to those of the controls throughout this period. Therefore therapeutic dosing with tiamulin is effective in treating *B. intermedia* infections, and maintaining egg production, but reinfection of treated birds can occur fairly rapidly. The fact that these birds became reinfected, and that there was prolonged colonisation of individual birds in other treatment groups, suggests that colonisation by *B. intermedia* does not stimulate a strong protective immunity. It would have been useful to isolate the tiamulin-treated birds from the others, to help determine whether the source of reinfection was from the other birds in the room, or was due to a proliferation of low numbers of organisms that were still present in the caeca of the treated birds. The large number of culture positive birds at slaughter in this tiamulin-treated group was difficult to explain, but may have been due to the antimicrobial interfering with other components of the intestinal flora, which normally provide some colonisation resistance against the spirochaete. Had these birds been kept longer, it is possible that their egg production would have started to fall as a result of recolonisation by the spirochaetes. The dose rate of tiamulin used in this experiment was based on that recommended for the treatment of swine dysentery, and was the same as the dose rate used by Stephens and Hampson (1999) for the treatment of a naturally infected broiler breeder flock. However, this dose may not be optimal for chickens. Continuous application of low concentrations of tiamulin in the feed or water might continue to suppress spirochaete growth whilst enhancing egg production.

Following the reintroduction of ZnB to the diet of 10 of the birds, there was a clearing of spirochaete infection after three weeks. Taken together with the results from the earlier part of the experiment, these findings suggest that 100 ppm ZnB in the diet is a useful measure for control/prophylaxis of AIS caused by *B. intermedia.* It is common to use between 40 to 50 rather than 100 ppm ZnB in adult chickens, and it would be interesting to determine the effects of such lower concentrations in flocks with confirmed AIS.

Colonisation by *B. intermedia* did not induce any consistent gross or macroscopic pathological change in the caeca of the birds, and, apart from a tendency for the contents to be more viscid and gassy, it is unlikely that colonisation would be detected at routine post-mortem. Spirochaetes could be seen in the lumen of the crypts, but there were no consistent associated histopathological changes. The mechanism(s) by which egg production losses occurred in colonised birds remains to be determined. The lack of characteristic histological change means that diagnosis of AIS caused by *B. intermedia* relies heavily on microbiological culture, preferably supplemented with PCR to enhance sensitivity of detection and allow speciation of the causal spirochaete.

4.1.2. Dietary enzyme and zinc bacitracin reduce colonisation of layer hens by the intestinal spirochaete *Brachyspira intermedia*

Summary

Brachyspira intermedia strain HB60 was used to experimentally infect 40 individually caged 22-week-old laying hens. Another 10 control birds were sham inoculated with sterile broth. All chickens received an experimental layer diet based on wheat. The infected birds were randomly divided into four groups of 10, with the diet for each group containing either 50 ppm zinc bacitracin (ZnB), 100 ppm ZnB, 256g/tonne of dietary enzyme (Avizyme®, 1302), or no additive. Birds were kept for six weeks after infection, and faecal excretion of *B. intermedia*, faecal water content, egg numbers, egg weights and body weights were recorded weekly. Control birds remained uninfected throughout the experiment. *B. intermedia* was isolated significantly less frequently from the groups of experimentally infected birds receiving ZnB at 50 ppm or Avizyme®, than those receiving 100 ppm ZnB, or no treatment. Infected birds had a transient increase in faecal water content in the week following challenge, but no other significant production differences were detected amongst the five groups of birds in subsequent weeks. It was not established how the ZnB at 50 ppm and the dietary enzyme reduced the ability of the spirochaete to colonise, but it may have been by bringing about changes in the intestinal microflora and/or the intestinal microenvironment.

Introduction

Very little has been published about means to control AIS. In the previous experiment we found that 100 ppm of zinc bacitracin (ZnB) in the diet helped control AIS caused by *B. intermedia* in experimentally infected layers. ZnB has been commonly used as a growth promoter/performance enhancer in both broilers and layers in overseas countries (Huyghebaert and de Groote, 1997). The current experiment was undertaken to verify this result, as well as to determine whether 50 ppm ZnB, a dose more usually used for growth promotion/performance enhancement, also would be effective in controlling the spirochaete. In addition, a dietary enzyme was investigated to determine whether it might assist in controlling AIS. The rationale for this approach was that soluble non-starch polysaccharides (sNSP) in the diet of pigs predispose to large intestinal proliferation of *Brachyspira hyodysenteriae* (Siba *et al.,* 1996; Pluske *et al.,* 1996, 1998) and *B. pilosicoli* (Hampson *et al*., 2000), and, since dietary enzymes degrade these sNSP, they potentially may aid in reducing spirochaete colonisation (Durmic *et al*., 2000). Furthermore, addition of xylanase to a wheat-based diet also has recently been shown to reduce colonisation with *Campylobacter jejuni* in experimentally infected chicks (Fernandez *et al*., 2000).

Materials and methods

Experimental birds

Fifty 18-week-old ISA-Brown layer hens were obtained from a commercial breeder. The birds were housed in an environmentally controlled (21-23^{o}C) facility, and randomly allocated to individual cages with mesh floors set in banks of 10 cages. Forty of the birds that were to be infected were placed in one room, and 10 uninfected control birds in an adjacent room. The birds were given 12 hours artificial light each day.

Experimental infection

Brachyspira intermedia, Western Australian chicken strain HB60, was obtained as a frozen stock culture from the collection held by the Reference Centre for Intestinal Spirochaetes at Murdoch University. This was thawed and grown in Kunkle's anaerobic broth medium (Kunkle *et al*., 1986). At 22 weeks of age, four weeks after they were obtained, 40 birds were orally inoculated via a crop tube with two mL of an actively growing culture, on three consecutive days. The broths contained approximately 10^8 bacterial cells per mL. The 10 uninfected control birds in the other room were sham-inoculated with sterile broth.

Diet and dietary treatments.

All the birds were fed a commercial wheat-based diet *ad libitum*, the composition of which is recorded in Table 11. The metabolisable energy, and macronutrient and micronutrient contents of the diet were sufficient to meet the nutrient needs of layer hens of this age (Leeson and Summers, 1997).The wheat was hammer milled through a 5mm screen. The birds to be experimentally infected were randomly allocated to four groups of 10. These groups received either the base diet, or the diet thoroughly mixed to contain 50 ppm ZnB (Alpharma Animal Health, Melbourne, Australia), 100 ppm ZnB, or 265g/tonne of a commercial dietary enzyme containing beta-xylanase and protease activity (Avizyme® 1302, Finnfeeds International, Marlborough, UK).

Body weights.

The birds were weighed weekly throughout the experiment.

Monitoring for spirochaetes in faeces.

Cloacal swabs were obtained weekly from each bird at the time they were handled for weighing, commencing prior to experimental inoculation with *B. intermedia*, and continuing for five weeks. The swabs were streaked onto selective Trypticase Soy Agar (BBL, Cockeysville, MD) supplemented with 5% defibrinated ovine blood, 400 µg/ml spectinomycin and 25 µg/ml each of colistin and vancomycin (Jenkinson and Wingar, 1981). Plates were incubated in an anaerobic environment generated by Gaspak *Plus* sachets (BBL), and growth was examined by phase

contrast microscopy after 5 and 10 days. To confirm the identity of spirochaetes present, bacterial growth on the primary plate was scraped off with a sterile glass slide, and an aliquot subjected to a polymerase chain reaction (PCR) specific for the 23S rRNA gene of *B. intermedia* (Suriyaarachchi et al., 2000).

Table 11: Composition of the basic layer mix diet

Ingredients	Composition (g/kg)
Wheat	505.3
Barley	100.0
Australian sweet lupins	60.0
Pollard	106.7
Meatmeal	133.3
Canola Oil	8.0
Limesand	78.7
Salt	2.0
Vital 400 (layer mineral/vitamin premix)	3.0
Carotine	0.25
Lysine	0.51
Methionine	2.27

Faecal moisture content

At the end of each week, aluminium foil was placed under the cage of each bird, and after one hour individual faecal samples were collected. An approximate one gram portion of the samples was weighed, dried to constant weight in a hot air oven, and the faecal moisture content calculated.

Egg production

Egg numbers and egg weights for each bird in each group were recorded daily, and accumulated to provide weekly records.

Post-mortem examination

When the birds were 28 weeks of age they were killed by cervical dislocation, and subjected to post-mortem examination. The caeca and colon were opened to look for evidence of gross changes, sections of one caecum placed in 10% buffered formalin for subsequent histological examination, and swabs taken from the other caecum for spirochaete culture. The fixed tissue was processed through to paraffin blocks, cut at 4µm and stained with haematoxylin and eosin.

Statistical analysis

Comparisons were made between the five groups of 10 birds. The numbers of birds in the four infected groups colonised by spirochaetes over the five weekly sampling periods post-infection

were compared using Chi-squared tests. Weekly and overall group bird weights, faecal moisture content, egg numbers and egg weights were compared using one-way analysis of variance. Means were compared using Fisher's protected least significant difference method, and significance accepted at the 5% level.

Results

Colonisation by spirochaetes

All faecal cultures carried out prior to inoculation of the birds were negative, and all faecal cultures of the 10 control birds were negative throughout the trial. In the five week period following inoculation, birds in all four inoculated groups shed spirochaetes in their faeces. Spirochaetes were also detected in the caeca at post mortem, one week after the last cloacal swabs were taken (Table 12). Results of PCR confirmed that these organisms were ***B. intermedia.*** Infected birds not receiving any supplements were most frequently colonised over the five weeks of sampling (31 of 50 bird sampling days), and birds receiving both 50 ppm ZnB and enzyme were less frequently colonised than this (respectively 11 of 50 bird sampling days; χ^2, 16.42, P <0.001, and 19 of 50 bird sampling days; χ^2, 5.76, P <0.025). Birds receiving 50 ppm ZnB also were less frequently colonised than birds receiving 100 ppm ZnB (χ^2, 4.96, P <0.05). The latter birds had 23 of 50 bird sampling days recorded as positive. No other differences between groups were significant.

Bird weights

Birds in all groups gained weight throughout the course of the experiment, and there were no significant group effects at any time.

Faecal moisture

Faecal moisture content was not significantly different between the groups, except in the first week following experimental inoculation (Table 13). At this sampling the faeces of the infected groups were all wetter than those of the uninfected control group, and, except for the group receiving 50 ppm ZnB, all these differences were significant.

Table 12: Faecal excretion of *B. intermedia* in the four groups of experimentally inoculated birds

Bird no.	Group	week 1[a]	week 2	week 3	week 4	week 5	Post-mortem[b]
11	Infected	+	-	-	-	+	-
12	Infected	+	+	+	+	+	+
13	Infected	-	-	+	+	+	+
14	Infected	+	-	+	+	+	+
15	Infected	-	+	+	+	+	+
16	Infected	-	+	+	+	+	+
17	Infected	-	-	-	-	+	+
18	Infected	+	+	-	-	+	-
19	Infected	-	-	-	-	+	-
20	Infected	-	+	+	+	+	+
21	ZnB 50	+	-	+	+	+	+
22	ZnB 50	-	-	-	-	+	+
23	ZnB 50	-	-	-	-	-	+
24	ZnB 50	+	+	-	-	-	+
25	ZnB 50	-	-	-	-	+	+
26	ZnB 50	+	-	-	-	-	+
27	ZnB 50	-	-	-	-	-	+
28	ZnB 50	-	-	-	-	-	+
29	ZnB 50	-	+	-	-	-	+
30	ZnB 50	+	-	-	-	-	+
31	ZnB 100	-	-	-	-	-	+
32	ZnB 100	-	+	+	+	+	+
33	ZnB 100	-	-	+	+	+	+
34	ZnB 100	+	-	+	-	+	+
35	ZnB 100	-	-	-	+	+	+
36	ZnB 100	-	+	+	+	+	+
37	ZnB 100	+	-	-	-	-	+
38	ZnB 100	-	-	-	-	+	+
39	ZnB 100	-	-	-	-	-	-
40	ZnB 100	+	+	+	+	+	+
41	Avizyme	-	+	+	+	-	+
42	Avizyme	-	-	+	+	-	-
43	Avizyme	-	-	+	-	-	+
44	Avizyme	-	+	-	-	+	+
45	Avizyme	+	-	-	+	-	+
46	Avizyme	-	-	+	+	-	+
47	Avizyme	-	+	-	-	+	+
48	Avizyme	-	-	+	+	+	+
49	Avizyme	-	-	-	-	+	+
50	Avizyme	-	-	+	-	-	+

[a]+, spirochaetes isolated; -, spirochaetes not isolated
[b]From caecal swabs

Table 13: Mean and standard error (in parentheses) faecal water content in the five groups of birds one week following experimental inoculation with *B. intermedia*

	Infected				
Control	No supplement	ZnB 50ppm	ZnB 100ppm	Avizyme®	*P* value
71.3[a] (2.6)	79.7[b] (2.0)	75.9[ab] (2.2)	80.3[b] (3.1)	79.9[b] (2.5)	0.0342

Means with different superscripts differ at the 5% level of significance

Egg numbers and total egg production

No significant differences were found in egg numbers or egg weights between the groups of birds at any time.

Post-mortem findings

The caecal size and contents varied between birds, with some caeca being small and having sparse contents, and others being large and full of contents of varying consistency. There were no consistent group effects. The mucosal surface of all the caeca examined were grossly normal, and histological examination failed to show any pathological changes. Spirochaetes were occasionally observed in the lumen and crypts, but no specific epithelial erosion or invasion by spirochaetes was recorded. *B. intermedia* was isolated from the caeca of all the experimentally infected birds, except three that did not receive any additive, and one bird each receiving 50 ppm ZnB and enzyme (Table 12).

Discussion

This experiment was successful in that it was possible to obtain *B. intermedia* colonisation of all the experimentally-infected untreated birds. Prolonged colonisation of individual birds also was observed, in some cases extending for six weeks from challenge to slaughter. Judging from the higher rates of colonisation detected by caecal swabs post-mortem compared to cloacal swabs a week earlier, cloacal swabs may not necessarily detect low levels of caecal colonisation. Nevertheless, cloacal swabs were useful for estimating comparative degrees of colonisation between the groups.

Despite the high level of colonisation in the infected untreated birds, apart from a transient significant increase in faecal water content in the week following inoculation, no significant production losses occurred. In two previous experiments, the same strain of *B. intermedia* (HB60) did cause increased faecal water content and/or reduced egg production in layers (Hampson and McLaren, 1999). The lack of significant effects on production in the current experiment in part may have been the result of its relatively short duration (six weeks following experimental challenge). Although there were also no consistent pathological changes in the caeca of colonised birds, this has been reported previously in birds that did suffer production losses, and the absence

of lesions can make field diagnosis of AIS difficult (Hampson and McLaren, 1999). It is possible that more subtle pathological changes could have been detected by using electron microscopy, but looking for these was not the object of the current experiment.

In agreement with our previous results, 100 ppm ZnB reduced colonisation with *B. intermedia* compared to untreated birds, although this reduction failed to reach significance in the current experiment. Unexpectedly, 50 ppm ZnB was significantly more effective than 100 ppm ZnB in reducing colonisation, and, although 50 ppm ZnB did not completely prevent colonisation, it had a highly significant protective effect. The reason for this difference in effectiveness against *B. intermedia* with the two levels of ZnB could be sought in its mode of action. ZnB is a bactericidal polypeptide antibiotic and is mainly active on Gram positive organisms. *B. intermedia* is Gram negative, and it is unlikely to be directly effected by the ZnB. On the other hand, an indirect effect is possible. ZnB is known to influence the overall composition of the intestinal microflora (Engberg *et al*., 2000). Since intestinal spirochaetes have a variety of complex interactions with the resident intestinal microflora, subtleties in the nature and extent of the alteration to the microflora and to the intestinal environment caused by the two levels of ZnB may explain the differences seen. Clearly, more work is required to examine the precise changes resulting from feeding different levels of ZnB. This may also require consideration of potential interactions between the level of ZnB and the specific diet used.

Consistent with our original hypothesis, addition of an enzyme compliment containing predominantly xylanase and protease to the wheat-based diet significantly reduced colonisation with *B. intermedia.* These results mirror those obtained in broiler chicks experimentally infected with *C. jejuni* (Fernandez *et al*., 2000). Again, the exact mechanisms by which this protective effect occurred in the current experiment are unclear, and require additional study. Enzymes reduce the viscosity of the intestinal contents by degrading dietary NSP (Bedford and Schulze, 1998). Fernandez *et al*. (2000) ascribed the protective effects they saw in broilers receiving xylanase to both a reduction in intestinal viscosity and alterations to mucin composition and carbohydrate expression of goblet cell glycoconjugates. Viscosity changes may be particularly pertinent, since when the viscosity of the intestinal contents was artificially increased in weaner pigs this stimulated proliferation of both enterotoxigenic *Escherichia coli* and *B. pilosicoli* (McDonald *et al*., 2000, 2001). Hence increased intestinal viscosity, and dietary factors related to it, may be important in predisposing to proliferation of pathogenic enteric bacteria, including spirochaetes (Hampson *et al*., 2001).

In view of the decline in availability of traditional growth promoting antimicrobials, the finding that dietary enzymes can reduce colonisation of chickens with intestinal spirochaetes is particularly significant. In practice, enzymes are already widely used for reducing wet litter

problems in poultry (Bedford and Morgan, 1996), and, in some cases, this outcome may be mediated through a reduction in colonisation by intestinal spirochaetes.

4.1.3. Zinc bacitracin enhances colonisation by the intestinal spirochaete *Brachyspira pilosicoli* in experimentally infected layer hens

Summary

Brachyspira pilosicoli strain CPSp1 isolated from a chicken in a broiler breeder flock in Queensland was used to experimentally infect 40 individually caged 22 week old laying hens. Another 10 birds were sham inoculated with sterile broth. All chickens received a commercial layer diet, but 10 infected birds had 50ppm zinc bacitracin (ZnB) incorporated in their food. Birds were kept for seven weeks, and faecal moisture, egg numbers, egg weights and body weights were recorded weekly. *B. pilosicoli* was isolated from the faeces of only three of the 30 inoculated birds receiving the diet without ZnB, whereas seven of the 10 inoculated birds receiving ZnB in their diet were colonised. This difference in colonisation rate was highly significant (P =<0.001). Dietary ZnB at 50 ppm therefore predisposed to colonisation by *B. pilosicoli.* Despite colonisation, no significant production differences were found between the birds in the three groups.

Introduction

There is little published information about control of AIS. Smit and colleagues (1998) described the treatment of naturally infected hens using 120 ppm of Ridzol S^{TM} (a 5-nitroimidazole compound) in the drinking water for six days. Treatment before the onset of lay prevented the negative effects of spirochaetes on egg production, but later treatment was less effective, and resulted in only a temporary improvement. This may have been due either to reinfection of the birds from contaminated litter, or to incomplete treatment of all the birds in the flock. Stephens and Hampson (1999) reported treating two meat breeder flocks with AIS using either lincospectin in the water for seven days at 50 mg per bird per day, or tiamulin at 25 mg per kg bodyweight in the water for five days. Following antimicrobial treatment, the condition of the birds in both flocks slowly improved. Initially the birds became spirochaete-free, but recolonisation slowly occurred over several months. In experiment 4.1.1. we showed that in-feed zinc bacitracin (ZnB) at 100 ppm inhibited colonisation with *B. intermedia* in experimentally infected layers, whilst a five day course of tiamulin at 25mg per kg body weight for five days resulted in a temporary clearance of colonisation.

The purpose of the present study was to evaluate the effects of 50 ppm ZnB in-feed, or a short course of treatment with either lincomycin or tiamulin, in layers held in controlled conditions and experimentally infected with an avian strain of *B. pilosicoli* which has been shown to cause

reduced egg production in experimentally infected broiler breeders (section 4.2.1). In the current experiment, colonisation rates by *B. pilosicoli* in experimentally inoculated birds not receiving ZnB were very low, so it was decided not to treat subgroups of these birds with lincomycin or tiamulin.

Materials and methods

Experimental birds

Fifty 18-week-old ISA-Brown layer hens were obtained from a commercial breeder. The birds were housed in an environmentally controlled (21-23^{o}C) facility, and randomly allocated to individual cages with mesh floors set in banks of 10 cages. Forty of the birds that were to be infected were placed in one room, and 10 uninfected control birds in an adjacent room. The birds were given 12 hours artificial light each day.

Experimental infection

Brachyspira (*Serpulina*) *pilosicoli*, Queensland chicken strain CPSp1, was obtained as a frozen stock culture from the collection held by the Reference Centre for Intestinal Spirochaetes at Murdoch University. This was thawed and grown in Kunkle's anaerobic broth medium (Kunkle *et al*., 1986). At 22 weeks of age, four weeks after they were obtained, 40 birds were orally inoculated via a crop tube with two mL of an actively growing culture, on three consecutive days. The broths contained approximately 10^8 bacterial cells per mL. The 10 uninfected control birds in the other room were sham-inoculated with sterile broth.

Diet

The birds were fed *ad libitum* on a commercial, vegetable-based layer diet provided by the commercial producer who supplied the birds. Ten of the experimentally infected birds received the same diet containing ZnB incorporated at 50 ppm.

Body weights

The birds were weighed weekly throughout the experiment.

Monitoring for spirochaetes in faeces

Cloacal swabs were obtained weekly from each bird at the time they were handled for weighing, commencing prior to experimental inoculation with *B. pilosicoli*. The swabs were streaked onto selective Trypticase Soy Agar (BBL, Cockeysville, MD) supplemented with 5% defibrinated ovine blood, 400 µg/ml spectinomycin and 25 µg/ml each of colistin and vancomycin (Jenkinson and Wingar, 1981). Plates were incubated in an anaerobic environment generated by Gaspak *Plus* sachets (BBL), and growth was examined by phase contrast microscopy after 5 and 10 days. Total bacterial growth on the primary plate was then scraped off with a sterile glass slide, and an aliquot

subjected to a polymerase chain reaction (PCR) specific for the 16S rRNA gene of *B. pilosicoli*, as previously described (Atyeo *et al.,* 1998; Mikosza *et al*., 1999).

Faecal moisture content

At the end of each week, aluminium foil was placed under the cage of each bird, and after one hour individual faecal samples were collected. Approximate one gram portions of the samples were weighed, then dried to constant weight in a hot air oven to determine the faecal moisture content.

Egg production

Egg numbers and egg weights for each bird in each group were recorded daily, and accumulated to provide weekly records.

Post-mortem examination

When the birds were 29 weeks of age they were killed by cervical dislocation, and subjected to post-mortem examination. The caeca and colon were opened to look for evidence of gross changes, sections of one caecum placed in 10% buffered formalin for subsequent histological examination, and swabs taken from the other caecum for spirochaete culture. The fixed tissue was processed through to paraffin blocks, cut at 4μm and stained with haematoxylin and eosin.

Statistical analysis

Comparisons were made between the 30 infected birds not receiving ZnB, the 10 infected birds receiving ZnB, and the 10 uninfected birds. The numbers of birds colonised by spirochaetes in the two infected groups were compared using a Chi-squared test. Weekly and overall group bird weights, faecal moisture content, egg numbers and egg weights were compared using one-way analysis of variance. Means were compared using Fisher's protected least significant difference method, and significance accepted at the 0.5% level.

Results

Colonisation by spirochaetes

All faecal cultures carried out prior to inoculation of the birds were negative, and all faecal cultures of the 10 control birds were negative throughout the trial. In the seven week period following inoculation, only three of the 30 experimentally inoculated birds on the diet without ZnB were culture positive (Table 14), and only one of these was positive on more than one sampling. Positive PCR signals were obtained for two other birds in the group, as well as the culture positive birds. Amongst the 10 birds receiving ZnB, seven were culture positive for *B. pilosicoli* (Table 14). One bird (no. 16) was positive in four samplings five weeks apart, and another (no. 15) on three

successive weeks. No additional new birds in this group were found to be positive by using PCR, but on week 28 PCR detected three colonised birds that were negative by culture alone. The colonisation rate of the birds receiving ZnB was highly significantly (χ^2, 14.4; P = <0.001) greater than that of the experimentally infected birds not receiving ZnB.

Table 14: Faecal excretion of *B. pilosicoli* amongst 40 experimentally-infected birds, 10 of which were receiving ZnB, as detected by direct culture and by PCR of total bacterial growth from the primary isolation plate.

Age of birds		ZnB		No ZnB	
(weeks)[a]		Culture	PCR	Culture	PCR
23	Bird number	12, 15	12, 15, 16	-	43
24		12, 15, 16	12, 15, 16	-	-
25		15, 16	15, 16	37	37, 45
26		14, 16, 17	14, 16, 17	-	-
27		19	19	34	34
28		13	13, 15, 16, 19	34, 43	34, 40, 43
29[b]		13, 16	13, 16	-	-

[a] Birds experimentally infected at 22 weeks of age

[b]Results from caecum at post-mortem examination

Bird weights

Birds in all groups gained weight throughout the course of the experiment, and there were no significant effects of infection or treatment on group body weights at any time.

Faecal Moisture

No statistically significant differences were found in the faecal moisture content of the birds in the three groups at any time during the experiment.

Egg numbers and total egg production

No significant differences were found in egg numbers or egg weights between the three groups of

birds at any point in the experiment.

Post-mortem findings

The caecal size and contents varied between birds, with some caeca being small and empty, and others being large and full of contents of varying consistency. There were no consistent group effects. The mucosal surface of all the caeca examined were grossly normal, and histological examination failed to show any pathological changes. No end-on attachment of spirochaetes to the lumenal surface of the caecal enterocytes was observed in any of the birds.

Discussion

This experiment gave several unexpected results. Colonisation rates amongst the 30 inoculated birds on the diet not containing ZnB were very low (10%), as assessed by faecal culture. Two additional birds were shown to be colonised by undertaking PCR on the total bacterial growth on the primary plate, and, as previously reported in pigs (Atyeo *et al*., 1998), this result helps to emphasise the usefulness of this PCR procedure for increasing the sensitivity of diagnosis of *B. pilosicoli* infections. The positive PCR results in the absence of spirochaete isolation presumably reflected a low level of colonisation in these two birds. Overall, for whatever reason, birds on this diet were not particularly susceptible to the strain of *B. pilosicoli* and the dose rate used. Consistent with this overall low rate of colonisation, no significant group effects were seen on any of the production parameters examined. As a consequence of the low rate of colonisation, it was not considered worthwhile treating subgroups of these birds with lincomycin or tiamulin, and this objective of the experiment was abandoned.

In contrast to the lack of colonisation amongst the 30 birds on the diet without ZnB, seven of the 10 birds on the same diet supplemented with 50 ppm ZnB became culture positive, and several birds were persistently colonised over a number of weeks. This difference in colonisation rate between the two inoculated groups was highly significant, and attributable to the presence of ZnB in the diet of the colonised birds. Based on our previous findings that 100 ppm ZnB inhibited colonisation of layers with *B. intermedia*, it was expected at the outset of the current experiment that the ZnB also would inhibit colonisation by *B. pilosicoli.* The diametrically opposite effects (inhibition of colonisation by *B. intermedia* compared to stimulation of colonisation by *B. pilosicoli*) were surprising. The differences appear to be specific to the spirochaete species rather than to the dose rate of ZnB used, since in a previous study *B. intermedia* colonisation in layers was inhibited by 50 ppm ZnB.

Zinc bacitracin is a bactericidal polypeptide antibiotic which mainly acts on Gram positive organisms. In overseas countries, but apparently not Australia, it is used as a growth promoter/performance enhancer in the diets of both broilers and laying hens (Huygbaert and Groote, 1997). When given in the diet it influences the composition of the intestinal microflora. For example, in broiler chicks it significantly reduces numbers of intestinal coliforms, *Clostridium perfringens* and *Lactobacillus salivarius* (Engberg *et al*., 2000). Intestinal spirochaetes are known to have a variety of complex interactions with different components of the intestinal microflora, and these may stimulate (Whipp *et al.,* 1979) or inhibit (Suenaga and Yamazaki, 1986) spirochaete growth. The most likely route by which the ZnB had its actions in the current experiment therefore is through altering the resident microflora, and consequently the intestinal environment. It is of interest that the diet also can modulate the dynamics of colonisation of pigs

with both *Brachyspira hyodysenteriae* (Siba *et al*., 1996) and *B. pilosicoli* (Hampson *et al*., 2000), again probably through its influence on the microflora and the intestinal environment. The current work suggests that components of the normal microflora of laying hens exert a degree of colonisation resistance against *B. pilosicoli*, and that this resistance can be removed by providing ZnB at 50 ppm in the diet. Further work is required to define the specific microflora involved in these interactions, and how it is altered by the ZnB. These sorts of interactions between *B. pilosicoli* and other components of the intestinal microflora may help to explain some of the variation in degree of colonisation and clinical signs observed in the field in infected birds, and in other animal species (including human beings).

Despite a high prevalence of colonisation (70%) amongst the birds receiving ZnB, no significant production effects were observed during the experimental period, and no characteristic pathological changes were observed in the caeca at slaughter. Using the same strain of *B. pilosicoli,* experimentally-infected broiler breeders have shown a reduction in egg production (section 4.2.1), and losses of egg production also have been recorded in flocks naturally infected with *B. pilosicoli* (Trampel *et al*., 1994; Stephens and Hampson, 1999). Possible reasons for the lack of effect in the current experiment include the relatively short duration of the experiment, the good husbandry and lack of stress that the experimental birds experienced, and other potential beneficial effects of the ZnB on health and production compensating for changes attributable to colonisation with *B. pilosicoli*. Although there was no end-on attachment of *B. pilosicoli* to the caecal epithelium in the two birds that were culture positive at slaughter, such attachment is not always a feature of colonisation (Trott and Hampson, 1998). The lack of histological change in the caeca of colonised birds emphasises the importance of using appropriate bacterial culture, preferably supplemented with PCR, in the diagnosis of *B. pilosicoli* infections.

The current findings have implications for the use of ZnB as a growth promoter/performance enhancer in chickens. By encouraging proliferation of pre-existing low numbers of *B. pilosicoli,* ZnB could increase excretion of the spirochaete, and potentially encourage disease expression in the flock itself. The possibility of increasing transmission of *B. pilosicoli* from chickens to other species, particularly human beings, also needs to be considered. Although the importance of *B. pilosicoli* as a zoonotic pathogen is still debated (Trott *et al.,* 1998), *B. pilosicoli* can cross species boundaries, and can cause disease in humans, particularly if they are immunocompromised (Trott *et al*., 1997; Trivett-Moore *et al.,* 1998; Trott *et al.,* 1998; Mikosza *et al.,* 2001). In conclusion, the possibility that ZnB can encourage shedding of *B. pilosicoli* from birds requires further investigation.

4.2. Experimental infection studies in broiler breeders

The studies on broiler breeder birds were conducted using the facilities of the Department of Primary Industries, Queensland, at the Animal Research Institute, Yeerongpily. The experiments were conducted with the approval of the Animal Ethics Review Committee, Southeast Region, Department of Primary Industries, Queensland. Two experiments were successfully conducted using *B. pilosicoli* to infect broiler breeders, and testing the efficacy of tiamulin and lincomycin to control the infections. The pathogenic potential of an avian isolate of *B. innocens* was also tested in the first experiment.

4.2.1. Experimental infection of broiler breeder hens with the intestinal spirochaete *Brachyspira pilosicoli* causes reduced egg production

Summary

The pathogenic potential of the anaerobic intestinal spirochaetes *Brachyspira pilosicoli* and *Brachyspira innocens* was evaluated in adult chickens. Thirty 17-week-old Cobb broiler breeder hens were individually caged in three equal groups of 10 birds. Control birds were sham inoculated with sterile broth medium. Birds in the other two groups were inoculated respectively with an isolate of *B. innocens* or of *B. pilosicoli*. Birds were monitored daily, and killed at 41 weeks of age. Infection had no consistent effect on body weight gain, but inoculation with *B. pilosicoli* resulted in a brief increase in faecal water content. *B. innocens* had no effect on egg production, but *B. pilosicoli* infection caused a delayed onset of laying, and a highly significant reduction in egg production over the first 11 weeks of lay. This study confirms that *B. pilosicoli* can cause serious egg production losses in adult chickens, whilst *B. innocens* is non-pathogenic.

Introduction

In contrast to the situation with *B. intermedia*, there have been no reports of studies using either *B. pilosicoli* or *B. innocens* to experimentally infect adult birds. *B. pilosicoli* is of particular comparative interest because it colonises many bird and animal species, including human beings (Trott *et al.,* 1997a, 1997b; Oxberry *et al.,* 1998; Trivett-Moore *et al*., 1998; Brooke *et al.,* 2001). The existence of cross-species transmission of *B. pilosicoli* has important implications for control of the infection in chicken flocks. Other intestinal spirochaete species, including *B. innocens*, also have been isolated from Australian chickens. *Brachyspira innocens* is generally considered to be a non-pathogenic spirochaete in conventional pigs (Kinyon and Harris, 1979), although porcine strains of this species have caused mucoid faeces and typhlocolitis in gnotobiotic pigs (Neef *et al.,* 1994).

The overall aim of the current study was to investigate the pathogenic potential of Australian chicken strains of *B. pilosicoli* and *B. innocens* in meat breeder hens, by inoculating them into birds from the same flock from which the strains were recovered.

Materials and Methods

Experimental birds

Thirty Cobb 500 broiler breeder females were obtained from a commercial producer at 13 weeks of age. The birds were placed in individual cages with mesh floors and egg roll-out trays. The cages were specially constructed to be large enough to accommodate breeder females. Each cage was provided with a waste tray for the collection of faeces. Clear plastic sheet was hung between cages to minimise the risk of transmission of infection between cages. The birds were kept in an air-conditioned room with temperatures varying between 17-23 ^{o}C. The day-length was set at 8 hours until 19 weeks of age, then gradually increased to 15 hours until 23 weeks of age and thereafter maintained at 16 hours. The birds were fed commercial diets supplied by the farm of origin, and these contained 50 ppm ZnB. They received a pullet developer diet until 19 weeks of age, then a pre-breeder ration. When egg production in the control group reached approximately 15%, all the birds were given a breeder production mix. Feed intake was restricted, with the birds being given 62 grams daily at 13 weeks of age, and this being gradually increased to a maximum of 165 grams per day by 27 weeks of age. Water was provided *ad libitum* by means of individual water bottles with nipple drinkers.

Spirochaete strains used for experimental infection

Brachyspira pilosicoli strain CPSp1 and *B. innocens* strain CPSi1, which were used to infect the birds, were isolated from breeders on the same farm from which the test birds originated. The strains had been isolated two years before the current study, during the course of a disease investigation, and their species identity had been confirmed through biochemical testing and polymerase chain reaction amplification of portions of their 16S rRNA and NADH oxidase genes (Stephens and Hampson, 1999). For experimental infection, the spirochaetes were grown to mid-log phase in Kunkle's broth (Kunkle *et al*., 1986). The inocula used to infect the birds contained approximately 10^8 bacterial cells per mL.

Experimental infection and monitoring of birds

The birds were acclimatised for four weeks. Over this period, individual faecal samples from each bird were taken weekly. These were cultured for spirochaetes on selective Trypticase Soy Agar (Micro Diagnostics, Australia), supplemented with 5% defibrinated bovine blood, 0.1% porcine mucin (Sigma, USA), 200 µg/ml spectinomycin and 6.25% µg/ml each of colistin and vancomycin. Plates were incubated in an anaerobic environment generated by Anaerogen sachets

(Oxoid, UK), and growth examined by dark field microscopy after 5 and 10 days. The species identity of isolates obtained during the course of the study was determined using a species-specific PCR protocol, as previously described (Stephens and Hampson, 1999).

After four weeks the birds were weighed and randomly assigned on the basis of body weight to one of three groups, each of ten birds. Birds in Group A (control group) were inoculated orally with one mL of sterile broth. Birds in Groups B and C were inoculated with one mL of a broth culture of either *B. innocens* or *B. pilosicoli* respectively.

Eggs from each bird were collected, counted and weighed every day. Once a week the birds were weighed, and individual faecal samples collected and cultured for intestinal spirochaetes. The faecal samples were weighed, dried to constant weight in a hot air oven, and the percentage faecal moisture calculated.

Post mortem examination

At 41 weeks of age the birds were killed by cervical dislocation, and subjected to post mortem examination. The caeca, ovaries and oviducts were examined grossly and sections fixed in 10% buffered formalin for subsequent histological examination. Caecal contents were cultured for intestinal spirochaetes.

Analysis

Weekly group bird weights and faecal moisture content were compared using one-way analysis of variance. Means were compared using Fisher's protected least significant difference method, and significance was accepted at the 0.5% level. Group egg production per week was compared using Chi square tests, except where values per cell were less than five, when Fisher's exact test was used in two by two contingency tables. Full egg production was assumed to be one egg/bird/day. The weights of the eggs produced were analysed by calculating a mean weight of eggs produced from each bird per week (ie total weight/number of eggs), then calculating group means of these bird means each week. These group means were compared by one-way analysis of variance.

Results

Body weights

There were few significant group differences in the body weights of the birds (Table 15). On week 26 the birds in group C were significantly heavier than those in the other two groups, and these birds were also significantly heavier than those in group B, but not in group A, on weeks 28, 29 and 32. Generally the birds gained weight as expected for the type and age of bird, and remained comparable with birds of the same batch in commercial production.

Colonisation

None of the birds were colonised with intestinal spirochaetes prior to the start of the experiment, and no control birds were colonised at any time. On week 18, one week following inoculation, *B. innocens* was isolated from one bird in group B. On week 19 an additional two birds in this group were positive for *B. innocens*, and all three remained positive for a further week. On week 21, four weeks after inoculation, only one of these three birds was positive. Thereafter and for the duration of the trial, none of the birds in group B were culture positive.

On week 18, one week following inoculation, *B. pilosicoli* was isolated from faecal samples from three birds in group C. These birds were positive the following week, but only one was positive the next week. All birds were culture negative by week 21, and remained so throughout the rest of the experiment.

Faecal water content

In weeks 19 and 20 the faecal moisture content of birds in group C was significantly higher than that of birds in group B, but not that of birds in group A (Table 16). At this time the faeces of birds in group C were 2-7% wetter than those in the other two groups. Thereafter there was no significant difference in the faecal moisture content between the groups, and there was no overall group difference in faecal moisture content.

Egg production

Birds in groups A and B both commenced laying at 23 weeks of age, however the onset of egg production in birds in group C was delayed for 2 weeks (Table 17). Ninety per cent of birds in groups A and B had commenced laying by 27 and 26 weeks of age respectively, but Group C did not reach this figure until the birds were 30 weeks of age.

Table 15: Group mean (± standard error) body weight of chickens (g) in the three experimental groups

Week	Group A (control)	Group B (*B. innocens*)	Group C (*B. pilosicoli*)	*P*-value
18	1645 (48)	1695 (44)	1635 (61)	0.7627
19	1755 (35)	1805 (45)	1765 (62)	0.3359
20	1845 (46)	1927 (37)	1855 (59)	0.5005
21	2010 (57)	2146 (42.62)	2055 (63)	0.2223
22	2345 (75)	2525 (52)	2355 (82)	1.508
23	2612 (49)	2655 (20)	2550 (95)	0.5021
24	2740 (62)	2790 (30)	2745 (108)	0.8710
25	2940 (62)	2970 (55)	2980 (120)	0.9410

26	3035 (76)[a]	3030 (77)[a]	3255 (46)[b]	0.0518
27	3310 (73)	3265 (70)	3439 (47)	0.1780
28	3390 (91)[ab]	3330 (81)[a]	3611 (57)[b]	0.0499
29	3330 (71)[ab]	3170 (93)[a]	3422 (54)[b]	0.0771
30	3460 (82)	3425 (88)	3644.44 (56)	0.1279
31	3555 (87)	3525 (95)	3644 (88)	0.6377
32	3655 (81)[ab]	3570 (77)[a]	3822 (60)[b]	0.0733
33	3720 (92)	3605 (82)	3850 (75)	0.1447
34	3635 (83)	3575 (87)	3816 (101)	0.1699
35	3745 (75)	3575 (96)	3669 (3)	0.3130
36	3735 (77)	3610 (93)	3787 (72)	0.3216
37	3700 (106)	3710 (96)	3812 (63)	0.6743
38	3725 (104)	3705 (97)	3856 (62)	0.5002
39	3780 (107)	3790 (100)	3856 (59)	0.8418
40	3840 (106)	3790 (97)	3887 (64)	0.7780

Means within a row having different superscripts differ at the 5% level of significance.

Egg production by birds in group C was significantly less than by birds in the other two groups from weeks 23 to 33, except in week 31. Thereafter no significant differences occurred. Overall birds in group C produced highly significantly ($P < 0.0001$) fewer eggs than birds in the other two groups.

Comparisons of the mean weights of eggs layed revealed no significant group effects either on a weekly basis or overall. Mean egg weights increased gradually from around 45 grams at start of lay to approximately 67 grams at week 41.

Bird health

Birds in group C had frothy brown faeces when sampled in the first two weeks following experimental inoculation, but not thereafter. The faeces of the birds in the other groups remained normal throughout.

Two birds in group C were euthanased, both one week after they were noticed to be depressed and off their feed. The first, killed in week 24, was diagnosed with tibial dyschondroplasia, and the second, killed in week 34, with hepatoma. No other abnormalities were found in these birds, and no spirochaetes were isolated from their caecae.

Post-mortem findings

At necropsy, the caeca of the birds from group C contained more gas and their contents were more frothy, fluid and considerably paler than those of the birds in the other two groups. No gross or histological lesions were found in the caecae, ovaries or oviducts of any of the birds. There was no evidence of end-on attachment of spirochaetes to the caecal epithelium, and no spirochaetes were isolated from any of the birds.

Table 16: Group mean (± standard error) percent faecal water content of chickens in the three experimental groups

Week	Group A (control)	Group B (*B. innocens*)	Group C (*B. pilosicoli*)	*P*-value
18	55.33 (1.78)	53.29 (2.33)	58.52 (1.15)	0.1421
19	55.94 (1.11)[a]	50.41 (2.36)[b]	57.56 (1.28)[a]	0.0143
20	54.0 (1.55)[a]	48.7 (1.29)[b]	55.4 (0.96)[a]	0.0024
21	56.2 (1.28)	53.8 (1.12)	53.7 (1.47)	0.3106
22	56.3 (1.12)	57.1 (0.96)	53.4 (1.31)	0.0681
23	58.4 (0.86)	57.2 (0.99)	56.8 (1.23)	0.5607
24	56.3 (1.16)	58.3 (0.70)	57.0 (0.79)	0.2815
25	55.3 (1.50)	58.6 (0.78)	56.4 (1.05)	0.1308
26	56.8 (1.56)	55.8 (1.62)	56.9 (1.25)	0.8484
27	56.0 (1.16)	54.9 (0.92)	55.4 (1.45)	0.7903
28	59.6 (0.84)	58.1 (0.81)	57.4 (1.30)	0.2804
29	55.4 (1.27)	54.7 (0.73)	56.4 (0.71)	0.4748
30	55.7 (0.92)	55.8 (0.83)	57.1 (1.19)	0.5688
31	57.9 (0.72)	58.0 (0.72)	58.9 (0.65)	0.5295
32	58.1 (0.64)[ab]	57.4 (1.14)[a]	60.5 (0.73)[b]	0.0564
33	57.1 (0.79)	58.4 (0.73)	58.5 (0.71)	0.3252
34	56.6 (1.06)	56.7 (0.86)	58.4 (0.81)	0.3092
35	57.1 (1.59)	57.6 (1.08)	56.0 (1.51)	0.7171
36	57.5 (1.13)	59.0 (0.96)	57.2 (0.85)	0.3948
37	58.1 (1.17)	58.1 (0.52)	57.8 (0.87)	0.9689
38	56.1 (0.79)	57.8 (0.90)	57.7 (0.74)	0.2661
39	56.8 (0.84)	57.4 (0.75)	58.3 (1.39)	0.5420
40	56.0 (1.22)	58.3 (0.79)	56.4 (1.06)	0.2630

Means within a row having different superscripts differ at the 5% level of significance.

Table 17: Total number of eggs produced per group of 10 chickens per week (maximum possible 70/week)

Age (weeks)	Group A (control)	Group B (*B. innocens*)	Group C (*B. pilosicoli*)	*P* value
23	1[a]	5[a]	0[b]	0.0272
24	4[a]	14[b]	0[c]	0.0001
25	15[a]	22[a]	5[b]	0.0015
26	22[a]	40[b]	5[c]	0.0000
27	31[a]	52[b]	17[c]	0.0000
28	44[a]	50[a]	26[b]	0.0014
29	49[a]	49[a]	33[b]	0.0525
30	64[a]	57[a]	37[b]	0.0000
31	55	46	44	0.2290
32	56[a]	57[a]	33[b]	0.0002
33	60[a]	58[a]	41[b]	0.0082
34	54	50	41	0.3066
35	51	48	40	0.5098
36	48	49	35	0.6473
37	52	45	34	0.1948
38	43	50	32	0.2222
39	48	48	41	0.8147
40	46	48	34	0.6519
41	44	35	34	0.2631
Total	787[a]	823[a]	532[b]	0.0000

Means within rows having different superscripts differ at the 5% level of significance.
Egg weights

Discussion

In Australia, natural infection with both *B. pilosicoli* and *B. innocens* has been reported in layer and broiler breeder flocks (Stephens and Hampson, 1999). The current study is the first report of experimental infection of commercial adult birds with these organisms.

Overall, the study showed that *B. innocens* is unlikely to have much pathogenic significance in adult birds. The presence of non-pathogenic spirochaetes in certain flocks however does complicate the diagnosis of avian intestinal spirochaetosis, and it requires the availability of reliable species-specific techniques, such as PCR, to differentiate pathogenic from non-pathogenic spirochaete isolates from chickens.

Following experimental inoculation of the birds in groups B and C with spirochaetes, only a small number of birds became culture positive, and this colonisation only persisted for a maximum of four weeks. The birds were given only a single challenge with a relatively small number of spirochaetes, and this may help account for the low proportion of birds which became culture positive. Interestingly, there was no evidence of cross-transmission of spirochaetes between or within groups of birds, but again this may have failed to arise due to the careful hygiene that was practised and the low level of colonisation overall. The low colonisation rate and relatively short duration of colonisation in individual birds may have been influenced by the inclusion of 50 ppm of ZnB in the commercial diets used. This antimicrobial was added to mimic the situation in the commercial flock from which the birds and the spirochaetes originated. It would be interesting to determine the extent of both colonisation and production losses that would result if the experiment were repeated without the inclusion of any growth promotants. Similarly, the good husbandry conditions practiced in the experiment may have minimised potential production problems that would occur in a more stressful commercial situation.

Faeces were only cultured weekly, and it is possible that transient colonisation of individual birds was missed. The culture detection method used also may not have been sufficiently sensitive to detect a low level of colonisation in some birds. Atyeo *et al*. (1998) have shown that the use of PCR on growth from primary plates can substantially increase detection rates for *B. pilosicoli* in pig faeces, achieving detection limits of around 10^4 cells/g faeces. More recently, *B. pilosicoli* has been detected in human faeces using PCR on DNA extracted directly from the faeces (Mikosza *et al.*, 2001). Future studies of *B. pilosicoli* in chickens should include use of such PCR protocols to improve the sensitivity of detection.

The experimental challenge with *B. pilosicoli* did not cause a reduction in body weight, and, on several occasions in the earlier part of the experiment, birds in group C were actually heavier than the other birds. This probably reflects the fact that the birds in group C were producing fewer eggs, and putting more energy into carcase body weight gain at this time.

The birds infected with *B. pilosicoli* showed a transient increase in faecal moisture in the first few weeks after infection, as well as having brown frothy faeces, but this effect did not persist. Had colonisation with the spirochaetes continued, faecal moisture content may have remained elevated. Although the increase in moisture content was only of a few percentage points, commercially this could be sufficient to cause problems with mechanical cleaning of manure, faecal staining of eggs, increased odour and attraction of flies.

The most striking and significant finding in the study was the delay in both onset of egg production and in reduced total egg production in the birds inoculated with *B. pilosicoli.* When these birds did produce eggs, however, these were not significantly lighter than those produced by birds in the other two groups. The major losses in production occurred in the first 11 weeks of lay, where in 10 of these weeks total egg production was significantly reduced in birds of group C. Over this 11 week period average egg productions per bird in groups A, B and C were 40.7, 45.0 and 26.8 respectively. Hence, over this initial period, birds in group C on average produced 14 less eggs than the birds in the control group, or had only two thirds of their level of production. In a commercial situation this loss would have an extremely serious economic impact. That such losses can occur was seen in Iowa, where a 100,000 bird layer flock infected with a spirochaete, which was later identified as *B. pilosicoli* (McLaren *et al*., 1997), was shown to have an overall 5% reduction in egg production (Trampel *et al.,* 1994).

No gross nor histological abnormalities were found in the caecae of any of the birds at post mortem examination. The lack of pathological changes at necropsy is not altogether surprising, because the birds were not colonised at this time, and they had had many weeks for any lesions to resolve. In future studies it would be useful to kill birds at the time they were culture positive. A practical outcome of the lack of gross and histological changes in the caecae is that it may not necessarily always be possible to diagnose infection with intestinal spirochaetes purely on pathological grounds. Diagnosis may have to be based on microbiological culture of appropriate samples from birds showing clinical signs. Previous studies have demonstrated that in flocks infected with spirochaetes and experiencing production problems, not all birds are necessarily positive on culture (Stephens and Hampson, 1999). Thus samples from a number of birds might have to be examined to enable a diagnosis to be made. Moreover, the anaerobic nature and fastidious growth requirements of intestinal spirochaetes make microbiological culture relatively difficult.

There was no obvious explanation for the delay and subsequent persistent reduction in egg production in the group of birds inoculated with *B. pilosicoli*, particularly as only three birds were confirmed to be colonised. It is possible that the other birds were colonised, but were not detected because samples were only tested once a week. Colonisation had ceased by the time egg production had started, and it could be speculated that greater losses may have occurred had colonisation persisted. At post mortem no evidence was found for abnormalities in the ovaries or oviducts, although again by the time the birds were killed they were laying normally. Possibly their intestinal function was temporarily impaired, resulting in reduced nutrient uptake, although this was not reflected in reduced body weight gain. The birds did develop transient frothy brown faeces with a slightly increased faecal water content, but did not have obvious diarrhoea. It was of

interest that the birds in this group had altered caecal contents at post-mortem, and this may reflect persistent subtle changes in the caecal microflora or in caecal function.

This investigation has confirmed that infection of commercial meat breeders with *B. pilosicoli* can significantly reduce egg production. Previously we have shown that infection with this organism is widespread in commercial layer and meat breeder flocks in Australia (Hampson and Stephens, 1999). Thus it would appear almost certain that the infection is causing important economic losses. Further experimental studies are needed to find reliable means to establish experimental colonisation, to examine the pathological basis of production losses, and to improve diagnosis and control of *B. pilosicoli* infections.

4.2.2. The use of tiamulin and lincomycin for the control of experimental avian intestinal spirochaetosis in broiler breeders infected with *Brachyspira pilosicoli*

Summary

Brachyspira pilosicoli strain CPSp1 isolated from a chicken in a broiler breeder flock in Queensland was used to experimentally infect 30 individually caged 22-week-old Cobb 500 broiler breeder hens. Another 10 birds were sham inoculated with sterile broth. The birds failed to become colonised. At 29 weeks of age they were transferred to a diet containing 50 ppm ZnB and were rechallenged with the *B. pilosicoli* strain at 32 weeks of age, weekly for five weeks. The majority of the birds then became colonised. Ten birds were then treated by crop tube with 25 mg/kg body weight tiamulin for five days, and 10 with 20mg/kg body weight lincomycin for five days. Both these treatments cleared the infection, whilst untreated birds remained infected. The results of this experiment support previous observations that ZnB at 50 ppm in the diet increases the susceptibility of birds to *B. pilosicoli* infection. The study also demonstrates the usefulness of both tiamulin and lincomycin for treatment of infection with *B. pilosicoli* in adult birds.

Introduction

This study was designed to test the efficacy of tiamulin and lincomycin for the treatment of AIS caused by *B. pilosicoli* in broiler breeder hens. Previously we had shown that these antimicrobials were effective for treatment of *B. intermedia* infections in layers. It was appropriate to test these antimicrobials with *B. pilosicoli,* since we had previously found that ZnB had opposite effects with the two spirochaete species.

Materials and Methods

Experimental birds

Forty Cobb 500 broiler breeder females were obtained from a commercial producer at 20 weeks of age. The birds were placed in individual cages with mesh floors and egg rollout trays. The cages were constructed so as to be large enough to accommodate breeder females. Each cage was provided with a waste tray for the collection of faeces. The birds were placed in four adjoining rooms. The birds were maintained under conditions of a common, controlled airflow with temperatures varying from an average minimum of 20.2^{o}C to an average maximum of 23.1^{o}C. The day-length was initially set at 8 hours, then gradually increased to 15 hours until 25 weeks of age and thereafter maintained at 16 hours.

Diets

The birds were initially fed a commercial diet containing no ZnB. From 29 weeks of age the birds were fed a commercial diet supplied by the farm of origin containing 50 ppm of ZnB. Feed intake was restricted, with the birds initially being given 100 grams daily at 20 weeks of age. This amount was increased to 110 g per day at 21 weeks of age, 115 g per day at 22 weeks of age and 120 g daily at 23 weeks of age. This feed rate was maintained until the birds reached 33 weeks of age, when the feed rate was reduced to 115 g per day. Water was provided *ad libitum* by means of water pipes running past the rear of the cages in each room and accessed individually by each bird in that room.

Spirochaete strains used for experimental infection

Brachyspira pilosicoli strain CPSp1, which was used to infect the birds, was isolated from breeders on the same farm from which the test birds originated. The strains had been isolated four years before the current study, during the course of a disease investigation, and their species identity had been confirmed through biochemical testing and polymerase chain reaction amplification of portions of their 16S rRNA and NADH oxidase genes (Stephens and Hampson, 1999). For experimental infection, the spirochaetes were grown to mid-log phase in Kunkle's broth (Kunkle *et al*., 1986). The inocula used to infect the birds contained approximately 10^8 bacterial cells per mL.

Experimental infection

The birds were acclimatised for two weeks. Over this period, individual faecal samples from each bird were taken three times. These were cultured for spirochaetes on selective Trypticase Soy Agar (Micro Diagnostics, Australia), supplemented with 5% defibrinated bovine blood, 0.1% porcine mucin (Sigma, USA), 200 µg/ml spectinomycin and 6.25% µg/ml each of colistin and vancomycin. Plates were incubated in an anaerobic environment generated by Anaerogen sachets (Oxoid, UK), and growth examined by dark field microscopy after 5 and 10 days.

After two weeks, that is, at age 22 weeks, the birds were weighed and randomly assigned on the basis of body weight to one of four groups, each of ten birds. Each group was placed in one of the four rooms. The thirty birds in Groups B, C and D were orally inoculated via a crop tube with two mL of culture of *B. pilosicoli* on three occasions, each 48 hours apart. At the same time, the 10 uninfected control birds were inoculated with sterile broth. This inoculation procedure was repeated a further three times at weekly intervals.

After six weeks, at age 32 weeks, the birds were again inoculated as above, the inoculation being repeated five times at weekly intervals. On this occasion, the inoculation was from a different vial of the same organism, *B. pilosicoli* strain CPSp1B, than that used above.

Treatment

When the birds were 39 weeks of age, the infected birds in group B were orally dosed daily on five consecutive days by crop tube with a 2 ml of a solution of tiamulin (Novartis Animal Health, Sydney, Australia) dissolved in sterile water, at a rate of 25 mg tiamulin per kg bodyweight per day. This was given approximately seven hours post feeding. Birds in Group C were orally dosed daily on five consecutive days by crop tube with a 2 ml of a solution of lincomycin (Pharmacia and Upjohn, Brisbane, Australia) dissolved in sterile distilled water, at a rate of 20 mg lincomycin per kg bodyweight per day.

Monitoring of faecal moisture and egg production

Eggs from each bird were collected, counted and weighed every day. Eggs were also scored for degree of faecal eggshell staining. Eggs were scored from zero for a clean shell to five for a heavily stained shell. Once a week the birds were weighed, and individual faecal samples collected and cultured for intestinal spirochaetes. The faecal samples were weighed, dried to constant weight in a hot air oven, and the percentage faecal moisture calculated.

Post mortem examination

At 43 weeks of age the birds were killed by cervical dislocation, and subjected to post mortem examination. The caeca, ovaries and oviducts were examined grossly and sections fixed in 10% buffered formalin for subsequent histological examination. Caecal contents were cultured for intestinal spirochaetes.

Statistical analysis

Weekly group bird weights and faecal moisture content were compared using one-way analysis of variance. Means were compared using Fisher's protected least significant difference method, and significance was accepted at the 0.5% level. Group egg production per week was compared using Chi square tests, except where values per cell were less than five, when Fisher's exact test was

used in two by two contingency tables. Full egg production was assumed to be one egg/bird/day. The weights of the eggs produced were analysed by calculating a mean weight of eggs produced from each bird per week (ie total weight/number of eggs), then calculating group means of these bird means each week. These group means were compared by one-way analysis of variance.

Results

Body Weights

Significant group differences in the body weights of the birds were limited (Table 18). At 24 weeks, two weeks after initial inoculation, the average weight of birds in Group A, the control group was significantly heavier than that of the birds in Groups B and C. At the ages of 25, 35 36 and 38 weeks the average weight of the birds in group A was significantly greater than those of the birds in Group B only. The average weight of birds in Group A was significantly greater than that of birds in both Groups B and C at age 40, 41 and 43 weeks.

Table 18: Group mean bodyweight of chickens (g) in the four experimental groups

Age (weeks)	Group A (control)	Group B (infected/tiamulin)	Group C (infected/lincomycin)	Group D (infected)	*P*-value
24	2745^{c}	2615^{a}	2625^{ab}	2680^{bc}	0.010
25	2865^{b}	2790^{a}	2800^{ab}	2840^{b}	0.049
35	3478^{b}	3255^{a}	3400^{ab}	3525^{b}	0.013
36	3528^{b}	3285^{a}	3430^{ab}	3530^{b}	0.038
38	3528^{b}	3305^{a}	3405^{ab}	3555^{b}	0.045
40	3606^{b}	3310^{a}	3360^{a}	3485^{ab}	0.011
41	3650^{b}	3335^{a}	3415^{a}	3495^{ab}	0.007
43	3661^{b}	3405^{a}	3435^{a}	3615^{ab}	0.044

Colonisation

None of the birds were colonised with intestinal spirochaetes prior to the start of the experiment and no control birds were colonised at any time throughout the experiment. Over the first six weeks following the original inoculations, no birds were found to be culture positive. At 33 weeks of age, one week following the commencement of the second set of inoculations, seven of the 10 birds in Group B were culture positive, as were five of the birds in group C and four of Group D. The following week, seven birds from Group B were again positive, as were six of group C and

seven of group D. Over the next two weeks, that is, at ages 35 and 36 weeks, the number of colonised birds in Group B rose to eight, while in group C and D, six and five birds respectively were positive. At 37 weeks, one week after final inoculation, there were six birds positive on culture in group B, six in Group C and three in group D. One week later, at 38 weeks of age, five birds in each of Groups B and C remained positive, as did four birds in Group D. At 39 weeks of age, the number of positive birds in Group B rose to six, while Groups C and D remained the same.

One day after the completion of treatment with tiamulin and lincomycin at age 39 weeks, all birds in both Groups B and C were negative on culture. All these birds remained negative on culture for the remaining four weeks of the trial, that is, until age 43 weeks. Three birds in Group D remained positive for a further three weeks, with two birds positive the last week of the trial.

Faecal water content

At 34 weeks of age, two weeks after the commencement of the second set of inoculations, the mean faecal moisture content of birds in Groups B and D was significantly greater than that of Groups A and C (Table 19). At 37 weeks, mean faecal moisture content of birds in Group D was significantly greater than that of Groups A and B, although not significantly greater than that of Group C, while at age 40 weeks, mean faecal moisture of Group B was significantly higher than that of Groups A and D. This was again the case the final week of the trial.

Table 19: Group mean faecal moisture of chickens (%) in the four experimental groups

Age (weeks)	Group A (control)	Group B (infected/tiamulin)	Group C (infected/lincomycin)	Group D (infected)	*P*-value
34	58.34^{a}	63.17^{b}	61.27^{ab}	62.59^{b}	0.012
37	54.96^{a}	54.80^{a}	57.70^{ab}	61.13^{b}	0.034
40	59.27^{ab}	63.45^{c}	62.81^{bc}	56.46^{a}	0.001
43	59.90^{bc}	60.57^{c}	56.59^{ab}	54.40^{a}	0.012

Egg production

There were no significant differences in egg numbers produced between the four groups of birds. At 38 and 39 weeks, two and three weeks respectively following the last inoculation, the mean weight of eggs produced by Group B was significantly less than that produced by either Group A or Group D, although it was not significantly less than Group C (Table 20). At 42 weeks of age, the mean weight of eggs produced by Group A was significantly greater than that produced by either Group B or group C.

Table 20: Group mean egg weights (g) in the four experimental groups

Age (weeks)	Group A (control)	Group B (infected/tiamulin)	Group C (infected/lincomycin)	Group D (infected)	*P*-value
38	67.44 [b]	62.02 [a]	63.62 [ab]	67.07 [b]	0.043
39	68.06 [c]	62.52 [a]	64.12 [ab]	66.23 [bc]	0.025
42	70.84	64.90	65.55	67.63	0.030

Faecal staining of eggs

There was a significant difference in mean degree of faecal staining of the eggshells of Group A compared with the other three groups at age 31 weeks, that is, six weeks after the completion of the initial inoculations (Table 21). Over the following week, the first week of the second set of inoculations, mean degree of faecal staining of eggs of Group A was significantly lower than that of either of Groups B and D. During the third and fourth weeks of the second set of inoculations, that is at age 34 and 35 weeks, faecal staining of eggshells of Group A, the control group, was significantly lower than that of the other three, infected groups. This was also the case at age 37 and 39 weeks.

Table 21: Group mean faecal staining of eggshells in the four experimental groups

Age (weeks)	Group A (control)	Group B (infected/tiamulin)	Group C (infected/lincomycin)	Group D (infected)	*P*-value
31	0.45 [a]	0.86 [b]	0.81 [b]	0.78 [b]	0.012
32	0.41 [a]	0.92 [ab]	0.80 [b]	1.02 [b]	0.032
34	0.51 [a]	0.95 [b]	1.05 [b]	1.01 [b]	0.021
35	0.50 [a]	0.90 [b]	1.22 [b]	0/94 [b]	0.002
37	0.57 [a]	1.04 [b]	1.15 [b]	1.24 [b]	0.007
38	0.50 [a]	0.90 [ab]	1.25 [b]	0.87 [ab]	0.013
39	0.48 [a]	1.28 [b]	1.35 [b]	1.15 [b]	0.001
40	0.49 [a]	0.97 [bc]	1.23 [c]	0.75 [ab]	0.020
41	0.56 [a]	1.33 [ab]	1.23 [bc]	0.87 [c]	0.008
42	0.54 [a]	0.78 [a]	1.29 [b]	0.87 [ab]	0.037

Bird health

One bird in group A was euthanased at 32 weeks of age, prior to the commencement of the second set of inoculations. The bird had been depressed and not standing, although still eating and drinking, for a period of one week. It was diagnosed with fatty liver haemorrhagic syndrome.

Silver impregnation stains of histological sections of the caecum failed to show any spirochaetes and caecal contents were negative on culture.

Post mortem examination

At necropsy, no gross nor histological lesions were found in the caecae, ovaries or oviducts of any of the birds. Silver impregnation stains revealed spirochaetes in the caecae of the two birds from Group D that had remained culture positive throughout. There was no evidence of end-on attachment of spirochaetes to the caecal epithelium. Caecal contents of all birds were negative on culture for spirochaetes with the exception of those of the two birds from Group D, both of which yielded heavy growth of spirochaetes. These were identified phenotypically as *B. pilosicoli*.

Discussion

This study was originally designed as a straightforward experiment to test the efficacy of tiamulin and lincomycin for the control of *B. pilosicoli* infection in broiler breeders, in the model described in 4.2.1. Unexpectedly, the birds failed to become colonised following experimental infection. In the original model, the birds received 100 ppm ZnB in their diet, but none was initially included in the diet in the current experiment. Again, consistent with our observation that 50 ppm ZnB greatly increases colonisation rates with *B. pilosicoli* in layers (section 4.1.3), in the current experiment the birds were readily colonised following reinoculation with the strain when they were then receiving 50 ppm ZnB in their diet. These findings therefore help to confirm that ZnB increases the susceptibility of adult chickens to infection with *B. pilosicoli.*

Once the birds became colonised with the *B. pilosicoli* strain, it became reasonably easy to demonstrate the efficacy of both tiamulin and lincomycin in removing the infection. In this study, perhaps because the treated birds were unable to physically contact each other and were housed in isolation from the untreated birds, they did not become reinfected as previously had happened in experimentally infected layers (section 4.1.1.), and in broiler breeders treated in the field (Stephens and Hampson, 1999). This suggests that reinfection is most likely to occur from other incompletely treated birds rather than from an endogenous residual infection, or from the immediate environment in the case of caged birds.

5. Implications

The fact that regular diagnoses of AIS were made in layer and breeder flocks throughout this two year project emphasises that infection with intestinal spirochaetes is a real problem to the Industries in Australia. The condition is undoubtedly going undiagnosed in many cases due to insufficient

awareness on the part of veterinarians and pathologists and a lack of expertise and facilities in diagnostic laboratories around Australia. Clearly both the Industries and the laboratories that service them need to be more aware of AIS as a possible differential diagnosis where wet litter and production drops are involved, and of what facilities are available for diagnosis. The molecular identification and strain typing techniques developed during this project will improve diagnostic capacity in Australia.

In general the spirochaete species remain susceptible to a range of antimicrobial agents, although resistance against tylosin was evident for a number of strains. The different spirochaete species tended to have a different range of sensitivities to some of the drugs tested. Clearly antimicrobial sensitivities need to be monitored on an ongoing basis.

The pathogenicity studies in adult birds confirmed that both *B. intermedia* and *B. pilosicoli* are capable of causing loss of production, although complex interactions which may modulate disease expression appear to occur between the spirochaetes and the intestinal microflora. In particular, in-feed zinc bacitracin increased the susceptibility of birds to *B. pilosicoli* infection, although it offered protection against *B. intermedia*. These differences emphasise the need for good diagnostic capacity to identify the spirochaete species involved in individual cases of AIS. Furthermore, research is required to understand the basis of these effects, so that improved control measures can be developed.

The study demonstrated that both tiamulin and lincomycin can be used to treat AIS. Both drugs are effective, but a problem arises from the potential for birds to become reinfected after treatment has ceased. Strategies for therapy should ensure all birds are adequately treated, and that the environment is cleaned and disinfected to reduce the opportunity for reinfection.

Finally, the observation that addition of xylanase to the diet can reduce colonisation by *B. intermedia* has important ramifications. The mechanisms behind this require further study, but presumably may be linked to changes in viscosity of the intestinal digesta. If this is the case, there are likely also to be dietary influences on AIS – although the possibility of different responses for *B. intermedia* and *B. pilosicoli* does need to be investigated.

6. Recommendations

The current findings should be disseminated to chicken producers and their veterinarians in Australia. In particular the potential for zinc bacitracin to increase susceptibility to *B. pilosicoli* should be made known. Similarly the potential beneficial effects of dietary xylanase preparations in reducing colonisation by *B. intermedia* in adult birds should be made known to the Industries.

Research funds should be made available for future research:

- to determine the mode of action of the xylanase in reducing colonisation by *B. intermedia*, and determining whether the same effect occurs with *B. pilosicoli*
- to investigate the mechanism whereby zinc bacitracin increases susceptibility to colonisation by *B. pilosicoli*
- to investigate the potential influence of diet on infection with intestinal spirochaetes in chickens.
- to study the epidemiology of the infections in depth: specifically it is necessary to find out how infection arises in a shed, how transmission occurs between birds and sheds, and what reservoirs of infection may exist. This can be supported by the methodologies for strain typing that have now been developed
- to investigate the potential for chicken isolates of *B. pilosicoli* to cause disease in humans

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